(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 December 2014 (04.12.2014)

WIPO | PCT

(10) International Publication Number
WO 2014/193888 A1

(51) International Patent Classification:
C07H 1/06 (2006.01) *C12P 19/56* (2006.01)
C12N 9/10 (2006.01)

(21) International Application Number:
PCT/US2014/039666

(22) International Filing Date:
28 May 2014 (28.05.2014)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

61/827,922	28 May 2013 (28.05.2013)	US
61/843,544	8 July 2013 (08.07.2013)	US
61/861,528	2 August 2013 (02.08.2013)	US
61/881,166	23 September 2013 (23.09.2013)	US
61/885,084	1 October 2013 (01.10.2013)	US
61/904,751	15 November 2013 (15.11.2013)	US
61/913,482	9 December 2013 (09.12.2013)	US
61/921,635	30 December 2013 (30.12.2013)	US
61/925,329	9 January 2014 (09.01.2014)	US
61/939,855	14 February 2014 (14.02.2014)	US
14/287,837	27 May 2014 (27.05.2014)	US

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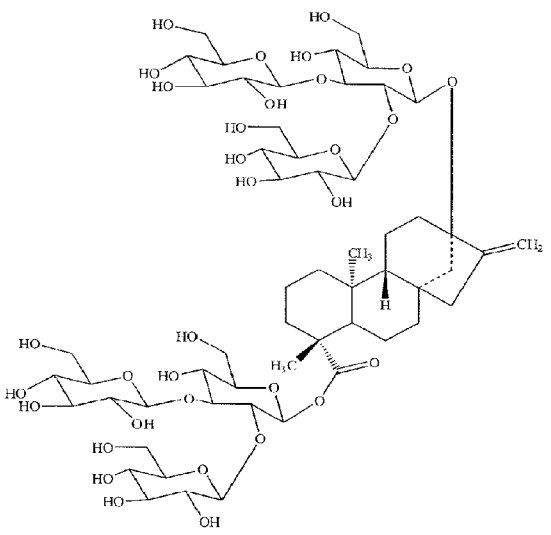
(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BN, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT,

[Continued on next page]

(54) Title: HIGH-PURITY STEVIOL GLYCOSIDES

FIG. 1



(57) Abstract: Methods of preparing highly purified steviol glycosides, particularly rebaudiosides *A, D* and *M* are described. The methods include utilizing recombinant microorganisms for converting various staring compositions to target steviol glycosides. In addition, novel steviol glycosides reb *D2* and reb *M2* are disclosed, as are methods of preparing the same. The highly purified rebaudiosides are useful as non-caloric sweetener in edible and chewable compositions such as any beverages, confectioneries, bakery products, cookies, and chewing gums.

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HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PA, PE, PG, PH, PL, PT, QA, RO, RS, RU, RW, SA, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TH, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) **Designated States** *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LR, LS, MW, MZ, NA, RW, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, RU, TJ, TM), European (AL, AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, RS, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, KM, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *with information concerning one or more priority claims considered void (Rule 26bis.2(d))*

— *with sequence listing part of description (Rule 5.2(a))*

HIGH-PURITY STEVIOL GLYCOSIDES

TECHNICAL FIELD

The present invention relates to a biocatalytic process for preparing compositions comprising steviol glycosides, including highly purified steviol glycoside compositions. The present invention also relates to novel steviol glycosides, methods for isolation of the same and uses for the novel steviol glycosides.

BACKGROUND OF THE INVENTION

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.

Steviol glycosides are a 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.

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

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

Additionally, there remains a need for novel steviol glycosides and methods of preparing and isolating the same.

SUMMARY OF THE INVENTION

The present invention provides a biocatalytic process for preparing a composition comprising a target steviol glycoside by contacting a starting composition comprising an organic substrate with a microorganism and/or biocatalyst, thereby producing a composition comprising a target steviol glycoside.

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 polyols or sugar alcohols, various carbohydrates.

The target steviol glycoside can be any steviol glycoside. In one embodiment, the target steviol glycoside is steviolmonoside, steviolbioside, rubusoside, dulcoside *B*, dulcoside *A*, rebaudioside *B*, rebaudioside *G*, stevioside, rebaudioside *C*, rebaudioside *F*, rebaudioside *A*, rebaudioside *I*, rebaudioside *E*, rebaudioside *H*, rebaudioside *L*, rebaudioside *K*, rebaudioside *J*, rebaudioside *M*, rebaudioside *M2*, rebaudioside *D*, rebaudioside *D2*, rebaudioside *N*, rebaudioside *O* or a synthetic steviol glycoside.

In one embodiment, the target steviol glycoside is stevioside.

In another embodiment, the target steviol glycoside is rebaudioside *A*.

In still another embodiment, the target steviol glycoside is rebaudioside *D*.

In yet another embodiment, the target steviol glycoside is rebaudioside *M* (also known as rebaudioside X).

The microorganism can be any microorganism possessing the necessary enzymes for converting the starting composition to target steviol glycosides.

The biocatalysts will comprise at least one enzyme for converting the starting composition to target steviol glycosides.

The biocatalysts can be located on the surface and/or inside the cell of the microorganism or can be secreted out of the microorganism.

The biocatalyst can be whole cell suspension, crude lysate or purified enzymes.

The biocatalyst can be in free form or immobilized to a solid support made from inorganic or organic materials.

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

In one embodiment the steviol biosynthesis enzymes include mevalonate (MVA) pathway enzymes.

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

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), l-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), l-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 etc.

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

In one embodiment, steviol biosynthesis enzymes and UDP-glucosyltransferases are produced in a microorganism. The microorganism 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.

In one embodiment, the UDP-glucosyltransferase is selected from group including UGT74G1, UGT85C2, UGT76G1, UGT91D2 and UGTs having substantial (>85%)

identity to these polypeptides as well as isolated nucleic acid molecules that code for these UGTs.

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

In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside to form stevioside. In a particular embodiment, the UDP-glucosyltransferase is UGT91D2.

In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside to form rebaudioside *A*. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1.

In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside *A* to form rebaudioside *D*. In a particular embodiment, the UDP-glucosyltransferase is UGT91D2. In another embodiment, the UGT is an improved variant of UGT91D2 with higher activity and/or selectivity produced by directed evolution.

In yet another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside *D* to form rebaudioside *M*. In a particular embodiment, the UDP-glucosyltransferase is UGT76G1. In another embodiment, the UGT is an improved variant of UGT76G1 with higher activity and/or selectivity produced by directed evolution.

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 the steviol glycoside substrate to the target steviol glycoside is carried out using catalytic amounts of UDP-glucosyltransferase and UDP-glucose (FIG. 3).

In one embodiment, the recycling catalyst is sucrose synthase.

In one embodiment, the recycling substrate is sucrose.

Optionally, the method of the present invention further comprises separating the target steviol glycoside from the starting 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.

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 reaction.

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.

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

Purified target steviol glycosides can be used in consumable products as a sweetener. Suitable consumer products include, but are not limited to, food, beverages, pharmaceutical compositions, tobacco products, nutraceutical compositions, oral hygiene compositions, and cosmetic compositions.

The present invention also provides novel steviol glycosides rebaudioside D2 (reb D2, isomer of rebaudioside D) and rebaudioside M2 (reb M2, isomer of rebaudioside M) , which are isomers of reb *D* and reb *M*, respectively. In one embodiment, isolated and purified reb *D2* is provided. In another embodiment, isolated and purified reb *M2* is provided. Reb *D2* and reb *M2* may also be present in any consumable products disclosed herein. In a particular embodiment, beverages comprising reb *D2* and/or reb *M2* are provided.

Methods of preparing reb *D2* and reb *M2* are also provided herein. Both are formed during the biotransformation of reb *A* to reb *D*. Reb *M2* is believed to form from biotransformation of reb *D2 in situ*.

In one embodiment, the present invention is a method for the preparation of a composition comprising reb *D2* comprising: (a) contacting a starting composition comprising reb *A* with an enzyme capable of transforming reb *A* to reb *D2*, UDP-glucose, and optionally UDP-glucose recycling enzymes, to produce a composition comprising reb *D2*, and (b) isolating the composition comprising reb *D2*.

In another embodiment, the present invention is a method for the preparation of a composition comprising reb *M2* comprising (a) contacting a starting composition comprising reb *D2* with an enzyme capable of transforming reb *D2* to reb *M2*, UDP-glucose, and optionally UDP-glucose recycling enzymes, to produce a composition comprising reb *M2*, and (b) and isolating the composition comprising reb *M2*.

A further method for the preparation of a composition comprising reb *M2* comprises (a) contacting a starting composition comprising reb *A* with an enzyme capable of transforming reb *A* to reb *D2*, UDP-glucose, and optionally UDP-glucose recycling enzymes, to produce a composition comprising reb *D2*, (b) optionally, isolating the composition comprising reb *D2*, (c) contacting the composition comprising reb *D2* with an enzyme capable of transforming reb *D2* to reb *M2*, UDP-glucose, and optionally UDP-glucose recycling enzymes to produce a composition comprising reb *M2*, and (d) isolating the composition comprising reb *M2*.

The composition can be further purified to provide reb *D2* or reb *M2* with purities greater than about 95% by weight on a dry basis.

BRIEF DESCRIPTION OF THE DRAWINGS

The accompanying drawings are included to provide a further understanding of the invention. The drawings illustrate embodiments of the invention and together with the description serve to explain the principles of the embodiments of the invention.

FIG. 1 shows the structure of reb *M*.

FIG. 2 shows the biocatalytic production of reb *M* from stevioside.

FIG. 3 shows the biocatalytic production of reb *A* from stevioside using the enzyme UGT76G1 and concomitant recycling of UDP to UDP glucose via sucrose synthase.

FIG. 4 shows the IR spectrum of reb *M*.

FIG. 5. shows the HPLC chromatogram of the product of the biocatalytic production of reb *M* from reb *D*, as detailed in Example 14. The peak with retention time of 24.165 minutes corresponds to unreacted reb *D*. The peak with retention time of 31.325 minutes corresponds to reb *M*.

FIG. 6. shows the HPLC chromatogram of purified reb *M* produced by biocatalysis from reb *D*.

FIG. 7 shows the HPLC chromatogram of a reb *M* standard.

FIG. 8 shows the HPLC chromatogram of co-injection of a reb *M* standard and reb *M* purified from biotransformation from reb *D*.

FIG. 9 shows an overlay of the ^{1}H NMR spectra of a reb *M* standard and reb *M* purified following biosynthesis from reb *D*.

FIG. 10 shows the HRMS spectrum of reb *M* purified following biocatalytic production from reb *D*.

FIG. 11 shows LC-MS analysis of semi-synthetic steviol glycoside mixture, Lot number CB-2977-106, showing TIC (A), MS of peak at 1.8 min (B), MS of reb *M2* peak at 4.1 min (C), MS of reb *D* peak at 6.0 min (D), MS of reb *D2* peak at 7.7 min (E), MS of peak at 9.4 min (F), MS of rebaudioside Apeak at 15.2 min (G), MS of peak at 16.5 min (H), and MS of peak at 18.3 min (I).

FIG. 12 shows the trace of semi-synthetic steviol glycoside mixture, Lot number CB-2977-106. Chromatogram gridlines are not homogeneous as the detector was re-calibrated 14 min following injection.

FIG. 13 shows HPLC analysis of semi-synthetic steviol glycoside mixture, Lot number CB-2977-106 (A), Isolated reb *M2* (B), isolated reb *D* (C) and isolated reb *D2* (D).

FIG. 14 shows the ^{1}H NMR spectrum of reb *D2* (500 MHz, pyridine-d_5).

FIG. 15 shows the ^{13}C NMR spectrum of reb *D2*(125 MHz, pyridine-d_5).

FIG. 16 shows an expansion of the ^{13}C NMR spectrum of reb *D2* (125 MHz, pyridine-d_5).

FIG. 17 shows the ^{1}H-^{1}H COSY Spectrum of reb *D2* (500 MHz, pyridine-d_5).

FIG. 18 shows the HSQC-DEPT spectrum of reb *D2*(500 MHz, pyridine-d_5).

FIG. 19 shows the HMBC spectrum of reb *D2*.

FIG. 20 shows an expansion of HMBC spectrum of reb *D2* (500 MHz, pyridine-d_5).

FIG. 21 shows the ^{1}H NMR spectrum of reb *M2*(500 MHz, D_2O).

FIG. 22 shows the ^{13}C NMR spectrum of reb *M2* (125 MHz, D_2O/TSP).

FIG. 23 shows an expansion of the ^{13}C NMR spectrum of reb *M2* (125 MHz, D_2O/TSP).

FIG. 24 shows the ^{1}H-^{1}H COSY spectrum of reb *M2* (500 MHz, D_2O).

FIG. 25 shows the HSQC-DEPT spectrum of reb *M2*(500 MHz, D_2O).

FIG. 26 shows the HMBC spectrum of reb *M2* (500 MHz, D_2O).

FIG. 27 shows an expansion of HMBC spectrum of reb *M2* (500 MHz, D_2O).

FIG. 28 shows another HMBC spectrum of reb *M2*.

FIG. 29 shows a ^{1}H NMR spectrum of reb *M2*.

FIG. 30 shows a ^{13}C NMR spectrum of reb *M2* .

FIG. 31 shows another ^{13}C NMR spectrum of reb *M2*.

FIG. 32 shows a ^{1}H-^{1}H COSY spectrum of reb *M2*.

FIG. 33 shows a HSQC-DEPT spectrum of reb *M2*.

FIG. 34 shows an HMBC spectrum of reb *M2*.

FIG. 35 shows another HMBC spectrum of reb *M2*.

FIG. 36 shows a 1D-TOCSY spectrum of reb *M2*.

FIG. 37 shows a 1D-TOCSY spectrum of reb *M2*.

FIG. 38 shows a 1D-TOCSY spectrum of reb *M2*.

FIG. 39 shows a 1D-TOCSY spectrum of reb *M2*.

FIG. 40 shows an HPLC (CAD) analysis.

FIG. 41 shows an HPLC (CAD) analysis.

FIG. 42 shows an HPLC (CAD) analysis.

FIG. 43 shows an HPLC (CAD) analysis.

FIG. 44 shows an HPLC (CAD) analysis.

FIG. 45 shows an HPLC (CAD) analysis.

FIG. 46 shows an HPLC (CAD) analysis.

FIG. 47 shows an HPLC (CAD) analysis.

FIG. 48 shows an HPLC (CAD) analysis.

FIG. 49 shows an HPLC (CAD) analysis.

FIG. 50 shows an HPLC (CAD) analysis.

FIG. 51 shows an HPLC (CAD) analysis.

FIG. 52 shows an HPLC (CAD) analysis.

FIG. 53 shows an LCMS chromatogram.

FIG. 54 shows an LCMS chromatogram.

FIG. 55 shows an LCMS chromatogram.

FIG. 56 shows an LCMS chromatogram.

FIG. 57 shows a reaction profile.

FIG. 58 shows an HPLC (CAD) analysis.

FIG. 59 shows an HPLC (CAD) analysis.

FIG. 60 shows an HPLC (CAD) analysis.

FIG. 61 shows an HPLC (CAD) analysis.

FIG. 62 shows an HPLC (CAD) analysis.

FIG. 63 shows an LCMS chromatogram.

FIG. 64 shows an HPLC (CAD) analysis.

FIG. 65 shows an HPLC (CAD) analysis.

FIG. 66 shows an HPLC (CAD) analysis.

FIG. 67 shows an HPLC (CAD) analysis.

FIG. 68 shows an HPLC (CAD) analysis.

FIG. 69 shows the results of an HPLC analysis.

DETAILED DESCRIPTION

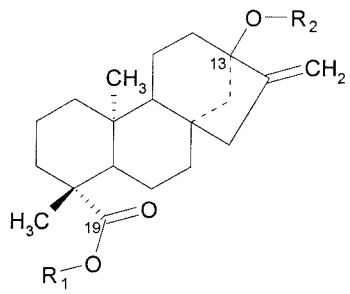
The present invention provides a biocatalytic process for preparing a composition comprising a target steviol glycoside by contacting a starting composition, comprising an organic substrate, with a microorganism and/or biocatalyst, thereby producing a composition comprising a target steviol glycoside.

One object of the invention is to provide an efficient biocatalytic method for preparing steviol glycosides, particularly stevioside, reb *E*, reb *A*, reb *D*, reb *D2*, reb *M*, and reb *M2* from various starting compositions.

As used herein, "biocatalysis" or "biocatalytic" refers to the use of natural or genetically engineered biocatalysts, such as cells, protein enzymes, to perform single or multiple step chemical transformations on organic compounds. Biocatalysis include fermentation, biosynthesis and biotransformation processes. Both, isolated enzyme and whole-cell biocatalysis methods, using biocatalysts in free as well as immobilized forms, are known in the art. Biocatalyst protein enzymes can be naturally occurring or recombinant proteins.

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, steviolbioside, rubusoside, dulcoside *B*, dulcoside *A*, rebaudioside *B*, rebaudioside *G*, stevioside, rebaudioside *C*, rebaudioside *F*, rebaudioside *A*, rebaudioside *I*, rebaudioside *E*, rebaudioside *H*, rebaudioside *L*, rebaudioside *K*, rebaudioside *J*, rebaudioside *M*, rebaudioside *M2,* rebaudioside *D*, rebaudioside *D2,* rebaudioside *N*, rebaudioside *O*, synthetic steviol glycosides, e.g. enzymatically glucosylated steviol glycosides and combinations thereof.

Chemical structures of steviol and its glycosides



Compound	R_1	R_2
Steviol	H	H
Steviolmonoside	H	β-Glc
Steviol monoglucosyl ester	β-Glc	H
Rubusoside	β-Glc	β-Glc
Steviolbioside	H	β-Glc-β-Glc (2→1)
Stevioside	β-Glc	β-Glc-β-Glc (2→1)
Rebaudioside A	β-Glc	β-Glc-β-Glc (2→1) | β-Glc (3→1)
Rebaudioside D	β-Glc-β-Glc (2→1)	β-Glc-β-Glc (2→1) | β-Glc (3→1)
Rebaudioside E	β-Glc-β-Glc (2→1)	β-Glc-β-Glc (2→1)
Rebaudioside M	β-Glc-β-Glc (2→1) | β-Glc (3→1)	β-Glc-β-Glc (2→1) | β-Glc (3→1)

(Glc=glucose)

Starting Composition

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.

In one embodiment, the starting composition is selected from the group consisting of polyols and various carbohydrates.

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.

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.

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.

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

In one embodiment, the starting composition is glycerol.

In another embodiment, the starting composition is glucose.

In still another embodiment, the starting composition is sucrose.

In yet another embodiment, the starting composition is starch.

In another embodiment, the starting composition is maltodextrin.

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

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, steviolbioside, rubusoside, dulcoside *B*, dulcoside *A*, rebaudioside *B*, rebaudioside *G*, stevioside, rebaudioside *C*, rebaudioside *F*, rebaudioside *A*, rebaudioside *I*, rebaudioside *E*, rebaudioside *H*, rebaudioside *L*, rebaudioside *K*, rebaudioside *J*, rebaudioside *M*,

rebaudioside *M2*, rebaudioside *D*, rebaudioside *D2*, rebaudioside *N* or rebaudioside *O,* or other glycoside of steviol.

In one embodiment, the target steviol glycoside is stevioside. In another embodiment, the target steviol glycoside is reb *A*. In still another embodiment, the target steviol glycoside is reb *E*. In yet another embodiment, the target steviol glycoside is reb *D*. In yet another embodiment, the target steviol glycoside is reb *D2*. In a further embodiment, the target steviol glycoside is reb *M*. In a still further another embodiment, the target steviol glycoside is reb *M2*.

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

In one embodiment, the present invention is a biocatalytic process for the production of reb *D*.

In yet another embodiment, the present invention is a biocatalytic process for the production of reb *D2*.

In still another embodiment, the present invention is a biocatalytic process for the production of reb *M*.

In a further embodiment, the present invention is a biocatalytic process for the production of reb *M2*.

Optionally, the method of the present invention further comprises separating the target steviol glycoside from the starting 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.

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 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 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 dry basis.

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

In another embodiment, when the target steviol glycoside is reb *M2*, the process described herein provides a composition having greater than about 90% reb *M2* content by weight on a dry basis. In another particular embodiment, when the target steviol glycoside is reb *M2*, the process described herein provides a composition comprising greater than about 95% reb *M2* content by weight on a dry basis.

In yet another embodiment, when the target steviol glycoside is reb *D*, the process described herein provides a composition greater than about 90% reb *D* content by weight on a dry basis. In another particular embodiment, when the target steviol glycoside is reb *D*, the process described herein provides a composition comprising greater than about 99% reb *D* content by weight on a dry basis.

In still another embodiment, when the target steviol glycoside is reb *D2*, the process described herein provides a composition greater than about 90% reb *D2* content by weight on a dry basis. In another particular embodiment, when the target steviol glycoside is reb *D2*, the process described herein provides a composition comprising greater than about 95% reb *D2* content by weight on a dry basis.

In a further embodiment, when the target steviol glycoside is reb *A*, the process described herein provides a composition comprising greater than about 90% reb *A* content by weight on a dry basis. In another particular embodiment, when the target steviol glycoside is reb *A*, the process described herein provides a composition comprising greater than about 95% reb *A* content by weight on a dry basis.

In a still further embodiment, when the target steviol glycoside is reb *E*, the process described herein provides a composition comprising greater than about 90% reb *E*

content by weight on a dry basis. In another particular embodiment, when the target steviol glycoside is reb *E*, the process described herein provides a composition comprising greater than about 95% reb *E* content by weight on a dry basis.

In a still further embodiment, when the target steviol glycoside is reb *I*, the process described herein provides a composition comprising greater than about 90% reb *I* content by weight on a dry basis. In another particular embodiment, when the target steviol glycoside is reb *I*, the process described herein provides a composition comprising greater than about 95% reb *I* content by weight on a dry basis.

In yet a further embodiment, when the target steviol glycoside is stevioside, the process described herein provides a composition comprising greater than about 90% stevioside content by weight on a dry basis. In another particular embodiment, when the target steviol glycoside is stevioside, the process described herein provides a composition comprising greater than about 95% stevioside content by weight on a dry basis.

Microorganism and biocatalysts

In one embodiment of present invention, a microorganism or biocatalyst is contacted with the starting composition to produce target steviol glycosides. The microorganism can be any microorganism possessing the necessary enzymes for converting the starting composition to target steviol glycosides. These enzymes are encoded within the microorganism's genome.

In one embodiment the microoganism may be, for example, *E.coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp,. *Bacillus* sp., *Yarrowia* sp. etc.

The enzymes can be located on the surface and/or inside the cell of the microorganism and/or can be secreted out in the medium by the microorganism.

The biocatalyst comprises at least one enzyme and can be whole cell suspension, crude lysate or purified enzyme.

The enzymes necessary for converting the starting composition to target steviol glycosides include the steviol biosynthesis enzymes and UDP-glycosyltransferases (UGTs). Optionally it may include UDP recycling enzyme(s). The UDP recycling enzyme can be sucrose synthase and the recycling substrate can be sucrose.

In one embodiment the steviol biosynthesis enzymes include mevalonate (MVA) pathway enzymes.

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

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), l-hydroxy-2-methyl-2(E)-butenyl 4-diphosphate synthase (HDS), l-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 etc.

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

In one embodiment, the microorganism is free. In another embodiment, the microorganism is immobilized. 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.

In one embodiment the microorganism is in aqueous medium, comprising water, and various components selected form group including carbon sources, energy sources, nitrogen sources, microelements, vitamins, nucleosides, nucleoside phosphates, nucleoside diphosphates, nucleoside triphosphates, organic and inorganic salts, organic and mineral acids, bases etc. Carbon sources include glycerol, glucose, carbon dioxide, carbonates,

bicarbonates. Nitrogen sources can include nitrates, nitrites, amino acids, peptides, peptones, or proteins.

In a particular embodiment, the medium comprises buffer. Suitable buffers include, but are not limited to, PIPES buffer, acetate buffer and phosphate buffer. In a particular embodiment, the medium comprises phosphate buffer.

In one embodiment, the medium can also include an organic solvent.

In one embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside, thereby producing stevioside. The UDP-glucosyltransferase may be, for example, UGT91D2.

In another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rubusoside, thereby producing rebaudioside *E*. The UDP-glucosyltransferase may be, for example, UGTSL2.

In still another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside *E*, thereby producing rebaudioside *D*. The UDP-glucosyltransferase may be, for example, UGT76G1.

In yet embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to stevioside, thereby producing rebaudioside *A*. The UDP-glucosyltransferase may be, for example, UGT76G1.

In a further embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside A, thereby producing rebaudioside *D* and/or rebaudioside *D2* and/or rebaudioside *M2*. The UDP-glucosyltransferase may be, for example, UGT91D2 or UGTSL2.

In another embodiment, the UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside *A* is selected from the following listing of GenInfo identifier numbers, preferably from the group presented in Table 1, and more preferably the group presented in 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
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
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 sativa*
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 sativa Japonica Group*
115471071	NP_001059134.1	*Oryza sativa Japonica Group*
116310985	CAH67920.1	*Oryza sativa Indica Group*
116788066	ABK24743.1	*Picea sitchensis*
122209731	Q2V6J9.1	*Fragaria x 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*
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*
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 x 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 sativa 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*	UGTSL
460386018	XP.004238697.1	*Solanum lycopersicum*	-
460409134	XP.004249995.1	*Solanum lycopersicum*	-
460410132	XP.004250485.1	*Solanum lycopersicum*	UGTSL2
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*	-

In yet another embodiment, the UDP-glucosyltransferase is any UDP-glucosyltransferase capable of adding at least one glucose unit to rebaudioside *D* to form rebaudioside *M* and/or rebaudioside *M2*. The UDP-glucosyltransferase may be, for example, UGT76G1.

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, i.e., a biocatalyst capable of UDP-glucose overproduction, and a recycling substrate, such that the conversion of the substrate steviol glycoside to the

target steviol glycoside is carried out using catalytic amounts of UDP-glucosyltransferase and UDP-glucose (FIG. 3).

In one embodiment, the UDP-glucose recycling catalyst is sucrose synthase.

In one embodiment, the recycling substrate is sucrose.

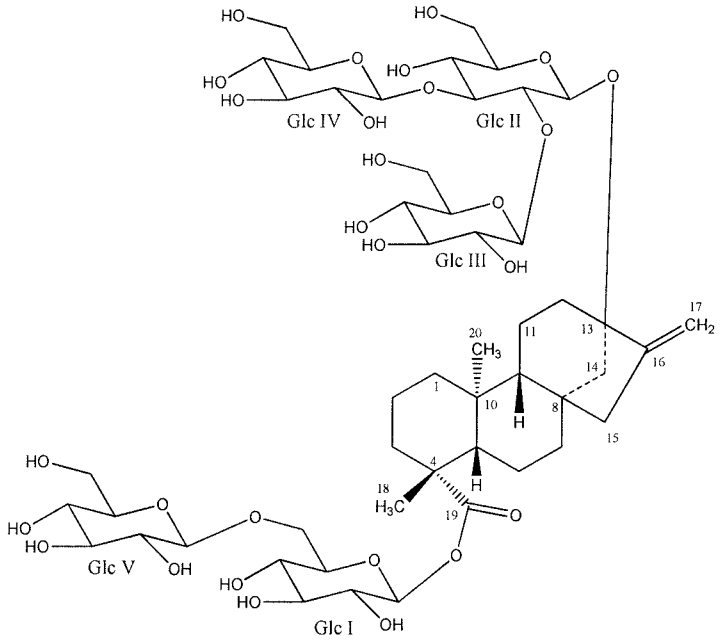
In one embodiment the biocatalyst comprises more than one UDP-glucosyltransferase.

In embodiment the biocatalyst comprises more than one UDP-glucosyltransferase and UDP-glucose recycling catalyst.

The target steviol glycoside is optionally purified from the resulting composition. 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.

Compounds and Methods

The present invention also provides isolated and highly purified reb *D2*. Reb *D2* is an isomer of reb *D* and has the following structure:



13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester]

In another embodiment, the present invention provides reb *D2* having a purity greater than about 95% by weight on an anhydrous basis, such as, for example, greater than about 96% by weight, greater than about 97% by weight, greater than about 98% by weight or greater than about 99% by weight.

In still another embodiment, the present invention provides reb *D2* having a purity greater than about 95% by weight in a steviol glycoside mixture, such as, for example, greater than about 96% by weight, greater than about 97% by weight, greater than about 98% by weight or greater than about 99% by weight.

The present invention also provides compositions comprising reb *D2*.

In one embodiment, the present invention provides a method for preparing reb *D2* comprising:

a. contacting a starting composition comprising reb A with an enzyme capable of transforming reb *A* to reb *D2*, UDP-glucose, and optionally UDP-glucose recycling enzymes, to produce a composition comprising reb *D2*; and

b. isolating the composition comprising reb *D2*.

In some embodiments, the enzyme capable of transforming reb *A* to reb *D2* is a UDP-glucosyltransferase, such as, for example, UGT91D2, UGTSL, UGTSL_Sc, UGTSL2 (GI No. 460410132 version XP_004250485.1), GI No. 460409128 (UGTSL) version XP_004249992.1, GI No. 115454819 version NP_001051010.1, GI No. 187373030, version ACD03249.1. GI No. 222619587 version EEE55719.1, GI No. 297795735 version XP_002865752.1 or EUGT11.

The enzyme capable of transforming reb *A* to reb *D2* can be immobilized or in a recombinant microorganism.

In one embodiment, the enzyme is immobilized. In another embodiment, the enzyme is in a recombinant microorganism.

In one embodiment, the microorganism is free. In another embodiment, the microorganism is immobilized. 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.

Suitable microorganisms include, but are not limited to, *E.coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp.

In one embodiment the microorganism is in an aqueous medium, comprising water, and various components selected form group including carbon sources, energy sources, nitrogen sources, microelements, vitamins, nucleosides, nucleoside phosphates, nucleoside diphosphates, nucleoside triphosphates, organic and inorganic salts, organic and mineral acids, bases etc. Carbon sources include glycerol, glucose, carbon dioxide, carbonates, bicarbonates. Nitrogen sources can include nitrates, nitrites, amino acids, peptides, peptones, or proteins.

In a particular embodiment, the medium comprises buffer. Suitable buffers include, but are not limited to, PIPES buffer, acetate buffer and phosphate buffer. In a particular embodiment, the medium comprises phosphate buffer.

In one embodiment the medium can also include an organic solvent.

In a particular embodiment, the enzyme is a UDP-glucosyltransferase capable of transforming reb *A* to reb *D2*.

In a more particular embodiment, the enzyme is selected from UGT91D2, UGTSL, UGTSL_Sc, UGTSL2 (GI No. 460410132 version XP_004250485.1), GI No. 460409128 (UGTSL) verison XP_004249992.1, GI No. 115454819 version NP_001051010.1, GI No. 187373030, version ACD03249.1. GI No. 222619587 version EEE55719.1, GI No. 297795735 version XP_002865752.1 or EUGT11 and UGTs having substantial (>85%) sequence identity to these.

In a still more particular embodiment, the enzyme is UGTSL2 or its improved variant produced by directed evolution and having higher activity.

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 reaction.

Isolation of reb *D2* from the reaction medium can be achieved by any suitable method to provide a composition comprising reb *D2*. Suitable methods include, but are not limited to, lysis, crystallization, separation by membranes, centrifugation, extraction (liquid or solid phase), chromatographic separation, HPLC (preparative or analytical) or a combination of such methods. In a particular embodiment, isolation can be achieved by lysis and centrifugation.

In some embodiments, isolation may result in a reb *D2* purity less than about 95% by weight on an anhydrous basis, and the composition may contain, e.g., steviol glycosides and/or residual reaction products. The composition comprising reb *D2* can be further purified to provide highly purified reb *D2*, i.e. reb *D2* having a purity greater than about 95% by weight on an anhydrous basis. In some embodiments, the compositions comprising reb *D2* can be further purified to provide reb *D2* having a purity greater than

about 96%, greater than about 97%, greater than about 98% or greater than about 99% by weight on an anhydrous basis.

Purification can be affected by any means known to one of skill in the art including, but not limited to, crystallization, separation by membranes, centrifugation, extraction (liquid or solid phase), chromatographic separation, HPLC (preparative or analytical) or a combination of such methods. In a particular embodiment, HPLC is used to purify reb *D2*. In a more particular embodiment, semi-preparative HPLC is used to purify reb *D2*.

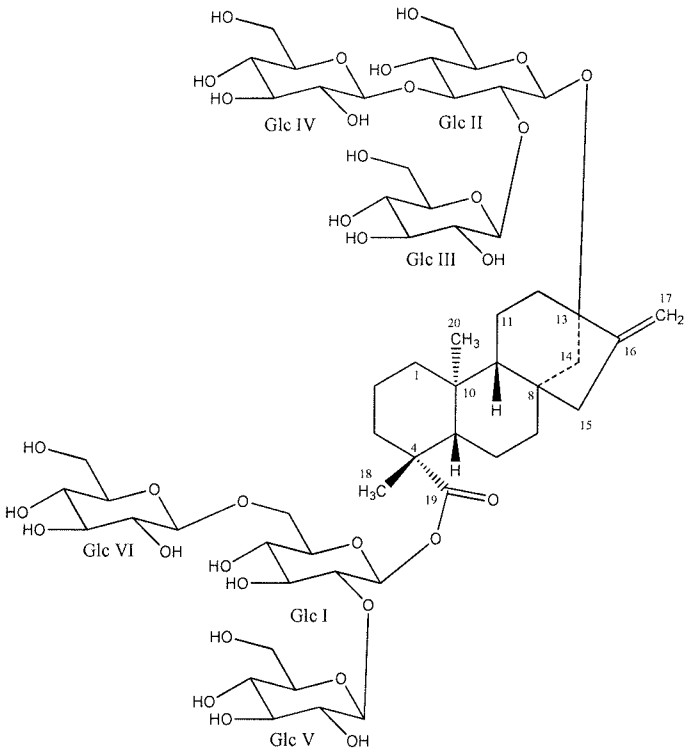
For example, a two-step semi-preparative HPLC purification can be used. The first step utilizes a C18 column with a mobile phase containing A (25% MeCN in water) and B (30% MeCN in water) with the following gradient:

Time (min)	%A	%B
0.0 – 5.0	100	0
20	20	80
25	20	80
30	100	0

The secondary step utilizes the same column and conditions, but with only an isocratic mobile phase: 20% MeCN in water.

Those of skill in the art will recognize that the particular column, mobile phases, injection volumes and other HPLC parameters can vary.

In one embodiment, the present invention provides isolated and highly purified reb *M2*. Reb *M2* is an isomer of reb *M* and has the following structure:



(13-[(2-*O*-β-D-glucopyranosyl-3-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(2-*O*-β-D-glucopyranosyl-6-*O*-β-D-glucopyranosyl-β-D-glucopyranosyl) ester])

In another embodiment, the present invention provides reb *M2* having a purity greater than about 95% by weight on an anhydrous basis, such as, for example, greater than about 96% by weight, greater than about 97% by weight, greater than about 98% by weight or greater than about 99% by weight.

In still another embodiment, the present invention provides reb *M2* having a purity greater than about 95% by weight in a steviol glycoside mixture, such as, for example, greater than about 96% by weight, greater than about 97% by weight, greater than about 98% by weight or greater than about 99% by weight.

In yet another embodiment, the present invention provides reb *M2* having a purity greater than about 95% by weight in a stevia extract, such as, for example, greater than about 96% by weight, greater than about 97% by weight, greater than about 98% by weight or greater than about 99% by weight.

The present invention also provides compositions comprising reb *M2*.

It has been found that reb *M2* is produced during biotransformation of reb *A* to reb *D*. As noted above, biotransformation of reb *A* to reb *D* also produces reb *D2*. Accordingly, in one embodiment, the present invention provides a method for preparing reb *M2* comprising:

a. contacting a starting composition comprising reb *A* and/or reb *D2* with an enzyme capable of transforming reb *A* and/or reb *D2* to reb *M2*, UDP-glucose, and optionally UDP-glucose recycling enzymes to produce a composition comprising reb *M2*; and

b. isolating a composition comprising reb *M2*.

Not wishing to be bound by theory, it is currently believed that the pathway begins with transformation of reb *A* to reb *D2*, followed by transformation of reb *D2* to reb *M2*. Accordingly, In one embodiment, the present invention provides a method for preparing reb *M2* comprising:

a. contacting a starting composition comprising reb *D2* with an enzyme capable of transforming reb *D2* to reb *M2*, UDP-glucose, and optionally UDP-glucose recycling enzymes to produce a composition comprising reb *M2*; and

b. isolating a composition comprising reb *M2*.

In yet another embodiment, a method for preparing reb M2 comprises:

a. contacting a starting composition comprising reb *A* with an enzyme capable of transforming reb *A* to reb *D2*, UDP-glucose, and optionally UDP-glucose recycling enzymes to produce a composition comprising reb *D2*;

b. optionally, isolating a composition comprising reb *D2*;

c. contacting the composition comprising reb *D2* with an enzyme capable of transforming reb *D2* to reb *M2*, UDP-glucose, and optionally UDP-glucose recycling enzymes to produce a composition comprising reb *M2*; and

d. isolating a composition comprising reb *M2*.

The enzyme can be a UDP-glucosyltransferase, such as, for example, UGT91D2, UGTSL, UGTSL_Sc, UGTSL2 (GI No. 460410132 version XP_004250485.1), GI No. 460409128 (UGTSL) version XP_004249992.1, GI No. 115454819 version NP_001051010.1, GI No. 187373030, version ACD03249.1. GI No. 222619587 version EEE55719.1, GI No. 297795735 version XP_002865752.1 or EUGT11 .

The enzyme can be immobilized or in a recombinant microorganism.

In one embodiment, the enzyme is immobilized. In another embodiment, the enzyme is in a recombinant microorganism.

In one embodiment, the microorganism is free. In another embodiment, the microorganism is immobilized. 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.

Suitable microorganisms include, but are not limited to, *E.coli, Saccharomyces* sp., *Aspergillus* sp., *Pichia* sp., *Bacillus* sp., *Yarrowia* sp.

In one embodiment the microorganism is in aqueous medium, comprising water, and various components selected form group including carbon sources, energy sources, nitrogen sources, microelements, vitamins, nucleosides, nucleoside phosphates, nucleoside diphosphates, nucleoside triphosphates, organic and inorganic salts, organic and mineral acids, bases etc. Carbon sources include glycerol, glucose, carbon dioxide, carbonates, bicarbonates. Nitrogen sources can include nitrates, nitrites, amino acids, peptides, peptones, or proteins.

In a particular embodiment, the medium comprises buffer. Suitable buffers include, but are not limited to, PIPES buffer, acetate buffer and phosphate buffer. In a particular embodiment, the medium comprises phosphate buffer.

In one embodiment the medium can also include an organic solvent.

In a particular embodiment, the enzyme is a UDP-glucosyltransferase capable of transforming reb *A* and/or reb *D2* to reb *M2* and is contained in E.*coli*.

In a more particular embodiment, the enzyme is selected from UGT91D2, UGTSL, UGTSL_Sc, UGTSL2 (GI No. 460410132 version XP_004250485.1), GI No. 460409128 (UGTSL) verison XP_004249992.1, GI No. 115454819 version NP_001051010.1, GI No. 187373030, version ACD03249.1. GI No. 222619587 version EEE55719.1, GI No. 297795735 version XP_002865752.1 or EUGT11.

In a still more particular embodiment, the enzyme is UGTSL2 or its improved variant produced by directed evolution and having higher activity.

In one embodiment, the target steviol glycoside reb M2 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 reaction.

Isolation of reb *M2* from the reaction medium can be achieved by any suitable method to provide a composition comprising reb *M2*. Suitable methods include, but are not limited to, lysis, crystallization, separation by membranes, centrifugation, extraction (liquid or solid phase), chromatographic separation, HPLC (preparative or analytical) or a combination of such methods. In a particular embodiment, isolation can be achieved by lysis and centrifugation.

In some embodiments, isolation may result in a reb *M2* purity less than about 95% by weight on an anhydrous basis, and the composition may contain, e.g., steviol glycosides and/or residual reaction products.

The composition comprising reb *M2* can be further purified to provide highly purified reb *M2*, i.e. reb *M2* having a purity greater than about 95% by weight on an anhydrous basis. In some embodiments, the compositions comprising reb *M2* can be further purified to provide reb *M2* having a purity greater than about 96%, greater than about 97%, greater than about 98% or greater than about 99% by weight on an anhydrous basis.

Purification can be affected by any means known to one of skill in the art including, but not limited to, crystallization, separation by membranes, centrifugation, extraction (liquid or solid phase), chromatographic separation, HPLC (preparative or analytical) or a combination of such methods. In a particular embodiment, HPLC is used to purify reb *M2*. In a more particular embodiment, semi-preparative HPLC is used to purify reb *M2*.

For example, a two-step semi-preparative HPLC purification can be used. The first step utilizes a C18 column with a mobile phase containing A (25% MeCN in water) and B (30% MeCN in water) with the following gradient:

Time (min)	%A	%B
0.0 – 5.0	100	0
20	20	80
25	20	80
30	100	0

The secondary step utilizes the same column and conditions, but with only an isocratic mobile phase: 20% MeCN in water.

Those of skill in the art will recognize that the particular column, mobile phases, injection volumes and other HPLC parameters can vary.

Purified steviol glycosides, prepared in accordance with the present invention, may be used in a variety of consumable products including, but not limited to, foods, beverages, pharmaceutical compositions, tobacco products, nutraceutical compositions, oral hygiene compositions, and cosmetic compositions.

The high purity reb *M* obtained in this invention, having a molecular weight of 1291.29, a molecular formula of $C_{56}H_{90}O_{33}$, CAS registry number 1220616-44-3, and the structure presented in FIG. 1, is in the form of a white and odorless powder. The

compound is about 200 times sweeter than sugar when compared to a 10% sucrose solution. The infrared absorption spectrum is shown in FIG. 4.

Other properties of the pure reb *M* compound include a melting point of 249-250°C, and a specific rotation of $[\alpha]_D^{25}$ -19.0° in 50% ethanol (C=1.0). The solubility of reb *M* in water is around 0.3%, and increases with an increase in temperature.

Reb *M* is soluble in diluted solutions of methanol, ethanol, n-propanol, and isopropanol. However, it is insoluble in acetone, benzene, chloroform, and ether.

Reb *M* obtained in accordance with the present invention is heat and pH-stable.

Highly purified target glycoside(s) particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* obtained according to this invention can be used "as-is" or in combination with at least one sweetener, flavor, food ingredient and/or combination thereof.

Non-limiting examples of flavors include lime, lemon, orange, fruit, banana, grape, pear, pineapple, mango, berry, bitter almond, cola, cinnamon, sugar, cotton candy and vanilla flavors and/or combination thereof.

Non-limiting examples of other food ingredients include at least one selected from flavors, acidulants, organic and amino acids, coloring agents, bulking agents, modified starches, gums, texturizers, preservatives, antioxidants, emulsifiers, stabilizers, thickeners and gelling agents and/or combination thereof.

Highly purified target glycoside(s) particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* obtained according to this invention can be prepared in various polymorphic forms, including but not limited to hydrates, solvates, anhydrous, amorphous forms and/or combination thereof.

Highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* 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.

Highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* as a sweetening compound may be employed as the sole sweetener, or it may be used together with at least one naturally occurring high intensity sweeteners such as stevioside, reb *A*, reb *B*, reb *C*, reb *D*, reb *E*, reb *F*, steviolbioside, dulcoside *A*, rubusoside, 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/or combination thereof.

In a particular embodiment, reb *D2* and/or reb *M2* can be used together in a sweetener composition comprising a compound selected from the group consisting of reb *A*, reb *B*, reb *D*, NSF-02, Mogroside V, erythritol and/or combinations thereof.

Highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* 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 the like.

Moreover, highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* can be used in combination with natural sweetener suppressors such as gymnemic acid, hodulcin, ziziphin, lactisole, and others. Reb *D*, reb *D2*, reb *M* and/or reb *M2* may also be combined with various umami taste enhancers. Reb *D*, reb *D2*, reb *M* and/or reb *M2* can be mixed with umami tasting and sweet amino acids such as glutamate, aspartic acid, glycine, alanine, threonine, proline, serine, glutamate, lysine and tryptophan.

Highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* 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.

Highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* 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.

Highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* may be combined with reduced calorie sweeteners such as D-tagatose, allulose, allose, L-sugars, L-sorbose, L-arabinose, and others.

Highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* 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.

Examples of carbohydrates which may be used in accordance with this invention include, but are not limited to, Psicose, turanose, allulose, allose, D-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.

Highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* 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.

Highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* 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, reb *D*, reb *D2*, reb *M* and/or reb *M2* 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.

Examples of consumable products in which highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* 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.

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

Moreover, the highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2* obtained in this invention may be used in dry or liquid forms. In one embodiment, a tabletop sweetener comprising reb *D2* is provided. In another embodiment, a tabletop sweetener comprising reb *M2* is provided.

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, reb *D*, reb *D2*, reb *M* and/or reb *M2* depends on the purpose of usage. As discussed above, it can be added alone or in combination with other compounds.

The present invention is also directed to sweetness enhancement in beverages using reb *D2*. The present invention is also directed to sweetness enhancement in beverages using reb *M2*. Accordingly, the present invention provides a beverage comprising a sweetener and reb *D2* and/or reb *M2* as a sweetness enhancer, wherein reb *D2* and/or reb *M2* is present in a concentration at or below their respective sweetness recognition thresholds.

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."

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.

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.

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.

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.

In another embodiment, the sweetener is erythritol.

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

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

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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%.

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.

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 reb *D2*, reb *M2* or a combination thereof, wherein reb *D2* and reb *M2* are present in a concentration at or below their sweetness recognition thresholds.

Addition of reb *D2* and/or reb *M2* 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%.

The following examples illustrate preferred embodiments of the invention for the preparation of highly purified target steviol glycoside(s), particularly, reb *D*, reb *D2*, reb *M* and/or reb *M2*. 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.

EXAMPLE 1

In-vivo production of UGT76G1

NcoI and NdeI restriction sides were added to the original nucleic sequence as described in Genbank accession no. AAR06912.1. After codon optimization the following nucleic sequence was obtained (SEQ ID 1):

CCATGGCCCATATGGAAAACAAAACCGAAACCACCGTTCGTCGTCGTCGCC
GTATTATTCTGTTTCCGGTTCCGTTTCAGGGTCATATTAATCCGATTCTGCAG
CTGGCAAATGTGCTGTATAGCAAAGGTTTTAGCATTACCATTTTTCATACCAA
TTTTAACAAACCGAAAACCAGCAATTATCCGCATTTTACCTTTCGCTTTATTCT
GGATAATGATCCGCAGGATGAACGCATTAGCAATCTGCCGACACATGGTCCG

CTGGCAGGTATGCGTATTCCGATTATTAACGAACATGGTGCAGATGAACTGC
GTCGTGAACTGGAACTGCTGATGCTGGCAAGCGAAGAAGATGAAGAAGTTA
GCTGTCTGATTACCGATGCACTGTGGTATTTTGCACAGAGCGTTGCAGATAG
CCTGAATCTGCGTCGTCTGGTTCTGATGACCAGCAGCCTGTTTAACTTTCAT
GCACATGTTAGCCTGCCGCAGTTTGATGAACTGGGTTATCTGGATCCGGATG
ATAAAACCCGTCTGGAAGAACAGGCAAGCGGTTTTCCGATGCTGAAAGTGAA
AGATATCAAAAGCGCCTATAGCAATTGGCAGATTCTGAAAGAAATTCTGGGC
AAAATGATTAAACAGACCAAAGCAAGCAGCGGTGTTATTTGGAATAGCTTTAA
AGAACTGGAAGAAAGCGAACTGGAAACCGTGATTCGTGAAATTCCGGCACC
GAGCTTTCTGATTCCGCTGCCGAAACATCTGACCGCAAGCAGCAGCAGCCT
GCTGGATCATGATCGTACCGTTTTTCAGTGGCTGGATCAGCAGCCTCCGAGC
AGCGTTCTGTATGTTAGCTTTGGTAGCACCAGCGAAGTTGATGAAAAAGATTT
TCTGGAAATTGCCCGTGGTCTGGTTGATAGCAAACAGAGCTTTCTGTGGGTT
GTTCGTCCGGGTTTTGTTAAAGGTAGCACCTGGGTTGAACCGCTGCCGGAT
GGTTTTCTGGGTGAACGTGGTCGTATTGTTAAATGGGTTCCGCAGCAAGAAG
TTCTGGCACACGGCGCAATTGGTGCATTTTGGACCCATAGCGGTTGGAATAG
CACCCTGGAAAGCGTTTGTGAAGGTGTTCCGATGATTTTTAGCGATTTTGGT
CTGGATCAGCCGCTGAATGCACGTTATATGAGTGATGTTCTGAAAGTGGGTG
TGTATCTGGAAAATGGTTGGGAACGTGGTGAAATTGCAAATGCAATTCGTCG
TGTTATGGTGGATGAAGAAGGTGAATATATTCGTCAGAATGCCCGTGTTCTG
AAACAGAAAGCAGATGTTAGCCTGATGAAAGGTGGTAGCAGCTATGAAAGCC
TGGAAAGTCTGGTTAGCTATATTAGCAGCCTGTAATAACTCGAG

After synthesis of the gene and subcloning into pET30A+ vector using NdeI and XhoI cloning sites, the UGT76G1_pET30a+ plasmid was introduced in *E. coli* Bl21(DE3) and *E. coli* EC100 by electroporation. The obtained cells were grown in petri-dishes in the presence of Kanamycin and suitable colonies were selected and allowed to grow in liquid LB medium (erlenmeyer flasks). Glycerol was added to the suspension as cryoprotectant and 400 µL aliquots were stored at -20 °C and at -80 °C.

The storage aliquots of *E. coli* BL21(DE3) containing the pET30A+_UGT76G1 plasmid were thawed and added to 30 mL of LBGKP medium (20 g/L Luria Broth Lennox; 50 mM PIPES buffer pH 7.00; 50 mM Phosphate buffer pH 7.00; 2.5 g/L glucose and 50 mg/L of Kanamycin). This culture was allowed to shake at 135 rpm at 30 °C for 8 h.

The production medium contained 60 g/L of overnight express instant TB medium (Novagen), 10 g/L of glycerol and 50 mg/L of Kanamycin. The medium was allowed to stir at 20 °C while taking samples to measure the OD and pH. The cultures gave significant growth and a good OD was obtained. After 40 h, the cells were harvested by centrifugation and frozen to yield 12.7 g of cell wet weight.

Lysis was performed by addition of Bugbuster Master mix (Novagen) and the lysate was recovered by centrifugation and kept frozen. Activity tests were performed with thawed lysate.

EXAMPLE 2

In-vitro production of UGT76G1

The S30 T7 High Yield Protein expression system kit from Promega was used. 4 μg of UGT76G1_pET30a+ plasmid from *E. coli* EC100 was mixed with 80 μL of S30 premix plus and 72 μL of S30 T7 extract was added. Nuclease-free water was added in order to obtain a total volume of 200 μL and the resulting solution was incubated for 2 h at 30°C. 180 μL was used in the catalytic test reaction.

EXAMPLE 3

In-vitro production of UGT91D2

NcoI and NdeI restriction sides were added to the original nucleic sequence as described in Genbank accession no. ACE87855.1. After codon optimization the following nucleic sequence was obtained (SEQ ID 2):

CCATGGCACATATGGCAACCAGCGATAGCATTGTTGATGATCGTAAACAGCT
GCATGTTGCAACCTTTCCGTGGCTGGCATTTGGTCATATTCTGCCGTATCTG
CAGCTGAGCAAACTGATTGCAGAAAAAGGTCATAAAGTGAGCTTTCTGAGCA
CCACCCGTAATATTCAGCGTCTGAGCAGCCATATTAGTCCGCTGATTAATGTT
GTTCAGCTGACCCTGCCTCGTGTTCAAGAACTGCCGGAAGATGCCGAAGCA
ACCACCGATGTTCATCCGGAAGATATTCCGTATCTGAAAAAAGCAAGTGATG
GTCTGCAGCCGGAAGTTACCCGTTTTCTGGAACAGCATAGTCCGGATTGGAT
CATCTATGATTATACCCATTATTGGCTGCCGAGCATTGCAGCAAGCCTGGGT
ATTAGCCGTGCACATTTTAGCGTTACCACCCCGTGGGCAATTGCATATATGG
GTCCGAGCGCAGATGCAATGATTAATGGTAGTGATGGTCGTACCACCGTTGA
AGATCTGACCACCCCTCCGAAATGGTTTCCGTTTCCGACCAAAGTTTGTTGG
CGTAAACATGATCTGGCACGTCTGGTTCCGTATAAAGCACCGGGTATTAGTG
ATGGTTATCGTATGGGTCTGGTTCTGAAAGGTAGCGATTGTCTGCTGAGCAA
ATGCTATCATGAATTTGGCACCCAGTGGCTGCCGCTGCTGGAAACCCTGCAT
CAGGTTCCGGTTGTTCCGGTGGGTCTGCTGCCTCCGGAAGTTCCGGGTGAT
GAAAAAGATGAAACCTGGGTTAGCATCAAAAAATGGCTGGATGGTAAACAGA
AAGGTAGCGTGGTTTATGTTGCACTGGGTAGCGAAGTTCTGGTTAGCCAGAC
CGAAGTTGTTGAACTGGCACTGGGTCTGGAACTGAGCGGTCTGCCGTTTGTT
TGGGCATATCGTAAACCGAAAGGTCCGGCAAAAAGCGATAGCGTTGAACTG
CCGGATGGTTTTGTTGAACGTACCCGTGATCGTGGTCTGGTTTGGACCAGCT
GGGCACCTCAGCTGCGTATTCTGAGCCATGAAAGCGTTTGTGGTTTTCTGAC
CCATTGTGGTAGCGGTAGCATTGTGGAAGGTCTGATGTTTGGTCATCCGCTG
ATTATGCTGCCGATTTTTGGTGATCAGCCGCTGAATGCACGTCTGCTGGAAG

ATAAACAGGTTGGTATTGAAATTCCGCGTAATGAAGAAGATGGTTGCCTGAC
CAAAGAAAGCGTTGCACGTAGCCTGCGTAGCGTTGTTGTTGAAAAAGAAGGC
GAAATCTATAAAGCCAATGCACGTGAACTGAGCAAAATCTATAATGATACCAA
AGTGGAAAAAGAATATGTGAGCCAGTTCGTGGATTATCTGGAAAAAAACACC
CGTGCAGTTGCCATTGATCACGAAAGCTAATGACTCGAG

After synthesis of the gene and subcloning into pET30A+ vector using NcoI and XhoI cloning sites, the UGT91D2_pET30a+ plasmid was introduced into *E. coli* EC100 by electroporation. The obtained cells were grown in the presence of Kanamycin and suitable colonies were selected and allowed to grow in liquid LB medium (erlenmeyer flasks). Glycerol was added to the suspension as cryoprotectant and 400 µL aliquots were stored at -20 °C and at -80 °C.

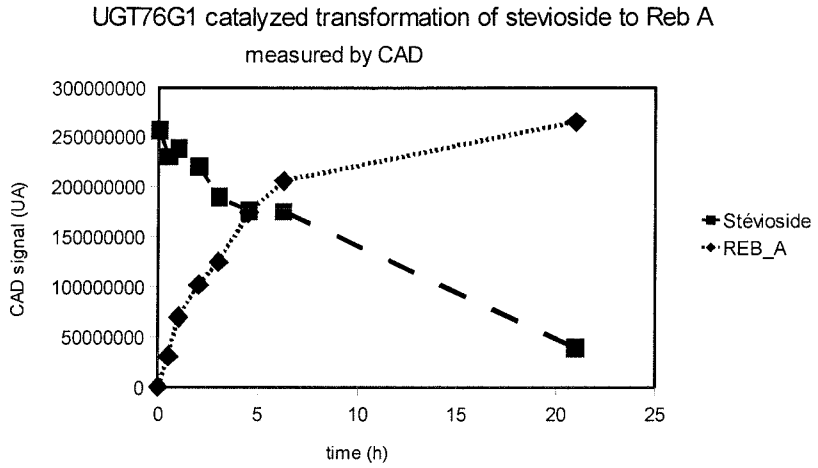
The S30 T7 High Yield Protein expression system kit from Promega was used for the in-vitro synthesis of the protein.

4 µg of UGT91D2_pET30a+ plasmid was mixed with 80 µL of S30 premix plus and 72 µL of S30 T7 extract was added. Nuclease-free water was added in order to obtain a total volume of 200 µL and the resulting solution was incubated for 2 h at 30°C. 5 µL was used for SDS-page analysis while the remaining 45 µL was used in the catalytic test reaction.

EXAMPLE 4

Catalytic reaction with in-vivo produced UGT76G1

The total volume of the reaction was 5.0 mL with the following composition: 50 mM sodium phosphate buffer pH 7.2, 3 mM $MgCl_2$, 2.5 mM UDP-glucose, 0.5 mM Stevioside and 500 µL of UGT76G1 thawed lysate. The reactions were run at 30 °C on an orbitary shaker at 135 rpm. For each sample, 460 µL of the reaction mixture was quenched with 40 µL of 2N H_2SO_4 and 420 µL of methanol/water (6/4). The samples were immediately centrifuged and kept at 10 °C before analysis by HPLC (CAD). HPLC indicated almost complete conversion of stevioside to rebaudioside A as seen in Figure 40.



EXAMPLE 5

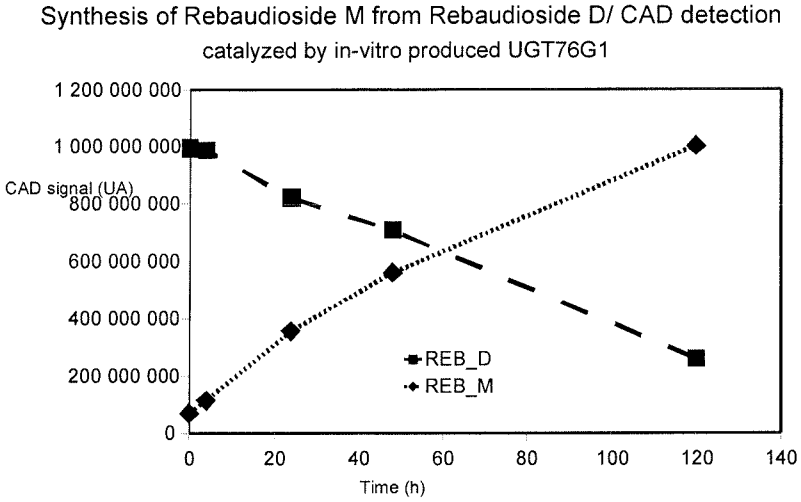
Catalytic reaction with in-vitro produced UGT91D2

The total volume of the reaction was 0.5 mL with the following composition: 50 mM sodium phosphate buffer pH 7.2, 3 mM $MgCl_2$, 3.8 mM UDP-glucose, 0.1 mM Rebaudioside A and 180 µL of in-vitro produced UGT91D2. The reactions were run at 30 °C on an orbitary shaker at 135 rpm. For each sample, 450 µL of reaction mixture was quenched with 45 µL of 2N H_2SO_4 and 405 µL of 60% MeOH. After centrifugation, the supernatant was analyzed by HPLC (CAD). HPLC indicated a 4.7% conversion of rebaudioside A to rebaudioside D after 120 h.

EXAMPLE 6

Catalytic reaction with in-vitro produced UGT76G1

The total volume of the reaction was 2 mL with the following composition: 50 mM sodium phosphate buffer pH 7.2, 3 mM $MgCl_2$, 3.8 mM UDP-glucose, 0.5 mM Rebaudioside D and 180 µL of in-vitro produced UGT76G1. The reactions were run at 30 °C on an orbitary shaker at 135 rpm. For each sample, 400 µL of reaction mixture was quenched with 40 µL of 2N H_2SO_4 and 360 µL of 60% MeOH. After centrifugation, the supernatant was analyzed by HPLC (CAD). HPLC indicated 80% conversion of rebaudioside *D* to rebaudioside *M* after 120 h as seen in Figure 41.



For examples 7 to 12, the following abbreviations were used:
LBGKP medium: 20 g/L Luria Broth Lennox; 50 mM PIPES buffer pH 7.00; 50 mM Phosphate buffer pH 7.00; 2.5 g/L glucose and 50 mg/L of Kanamycin or Ampicillin
LB medium: (20 g/L Luria Broth Lennox)

EXAMPLE 7

Preparation and activity of UGT76G1 prepared by pET30a+ plasmid and BL21 (DE3) expression strain

The pET30a+_UGT76G1 plasmid was transformed into BL21(DE3) expression strain (Lucigen *E. Cloni*® EXPRESS Electrocompetent Cells). The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Kanamycin. Suitable colonies were selected and allowed to grow in liquid LBGKP medium containing Kanamycin. Glycerol was added and 400 µL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 30 mL of LBGKP medium. This culture was allowed to shake at 30°C for 8 h. and subsequently used to inoculate 400 mL of production medium containing 60 g/L of "Overnight express instant TB medium" (Novagen, reference 71491-5), 10 g/L of glycerol and 50 mg/L of Kanamycin. The medium was allowed to stir at 20°C while taking samples to measure the OD (600 nm) and pH. After 40 h, the cells were harvested by centrifugation and frozen. The obtained cell wet weight was 10.58 g.

3.24 g of obtained pellet was lysed by addition of 8.1 mL of "Bugbuster Master mix" (Novagen, reference 71456) and 3.5 mL of water. The lysate was recovered by centrifugation and kept frozen.

EXAMPLE 8

Preparation and activity of UGT76G1 prepared by pET30a+ plasmid and Tuner (DE3) expression strain

The pET30a+_UGT76G1 plasmid was transformed into Tuner (DE3) expression strain (Novagen Tuner^{tm} (DE3) Competent cells) by heat shock treatment. The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Kanamycin. Suitable colonies were selected and allowed to grow in liquid LBGKP medium containing Kanamycin). Glycerol was added and 400 µL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 100 mL of LB medium containing 50 mg/L of Kanamycin. This culture allowed to shake at 30°C for 15 h. 4.4 mL of this culture was used to inoculate 200 mL of production medium containing LB. This medium was allowed to stir at 37°C until an OD (600 nm) of 0.9 was obtained, after which 400 µL of a 100 mM IPTG solution was added and the medium was allowed to stir at 30 °C for 4 h. The cells were harvested by centrifugation and frozen. The obtained cell wet weight was 1.38 g.

The obtained pellet was lysed by addition of 4.9 mL of "Bugbuster Master mix" (Novagen, reference 71456) and 2.1 mL of water. The lysate was recovered by centrifugation and kept frozen.

EXAMPLE 9

Preparation and activity of UGT76G1 prepared by pMAL plasmid and BL21 expression strain

After subcloning the synthetic UGT76G1 gene into the pMAL plasmid using NdeI and SalI cloning sites, the pMAL_UGT76G1 plasmid was transformed into BL21 expression strain (New England Biolabs BL21 Competent *E. coli*) by heat shock treatment. The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Ampicillin. Suitable colonies were selected and allowed to grow in liquid LBGKP medium containing Ampicillin). Glycerol was added and 400 µL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 30 mL of LBGKP medium. This culture was allowed to shake at 30°C for 8 h. and subsequently used to inoculate 400 mL of production medium containing 60 g/L of "Overnight express instant TB medium"

(Novagen, reference 71491-5), 10 g/L of glycerol and 50 mg/L of Ampicillin. The medium was allowed to stir at 20 °C while taking samples to measure the OD and pH. After 40 h, the cells were harvested by centrifugation and frozen. The obtained cell wet weight was 5.86 g.

2.74 g of obtained pellet was lysed by addition of 9.6 mL of "Bugbuster Master Mix" (Novagen, reference 71456) and 4.1 mL of water. The lysate was recovered by centrifugation and kept frozen.

EXAMPLE 10

Preparation and activity of UGT76G1 prepared by pMAL plasmid and ArcticExpress expression strain

The pMAL_UGT76G1 plasmid was transformed into ArticExpress expression strain (Agilent ArcticExpress competent cells) by heat shock treatment. The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Ampicillin and Geneticin. Suitable colonies were selected and allowed to grow in liquid LBGKP medium containing of Ampicillin and Geneticin. Glycerol was added and 400 µL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 30 mL of LBGKP medium (containing Ampicillin and Geneticin). This culture was allowed to shake at 30°C for 8 h. and subsequently used to inoculate 400 mL of production medium containing 60 g/L of "Overnight express instant TB medium" (Novagen, reference 71491-5), 10 g/L of glycerol and 50 mg/L of Ampicillin. The medium was allowed to stir at 12°C while taking samples to measure the OD (600 nm) and pH. After 68 h, the cells were harvested by centrifugation and frozen. The obtained cell wet weight was 8.96 g.

2.47 g of the obtained pellet was lysed by addition of 8.73 mL of "Bugbuster Master Mix" (Novagen, reference 71456) and 3.79 mL of water. The lysate was recovered by centrifugation and kept frozen.

EXAMPLE 11

Preparation and activity of UGT76G1 prepared by pCOLDIII plasmid and ArcticExpress expression strain

After subcloning the synthetic UGT76G1 gene into the pCOLDIII plasmid using NdeI and XhoI cloning sites, the pCOLDIII_UGT76G1 plasmid was transformed into

ArcticExpress expression strain (Agilent ArcticExpress competent cells) by heat shock treatment. The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Ampicillin and Geneticin. Suitable colonies were selected and allowed to grow in liquid LBGKP medium containing Ampicillin and Geneticin. Glycerol was added and 400 μL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 30 mL of LBGKP medium (containing Ampicillin and Geneticin). This culture was allowed to shake at 30°C for 8 h. and subsequently used to inoculate 400 mL of production medium containing 60 g/L of "Overnight express instant TB medium" (Novagen, reference 71491-5), 10 g/L of glycerol and 50 mg/L of Kanamycin. The medium was allowed to stir at 12°C while taking samples to measure the OD (600 nm) and pH. After 63 h, the cells were harvested by centrifugation and frozen. The obtained cell wet weight was 6.54 g.

2.81 g of the obtained pellet was lysed by addition of 9.8 mL of "Bugbuster Master Mix" (Novagen, reference 71456) and 4.2 mL of water. The lysate was recovered by centrifugation and kept frozen.

EXAMPLE 12

Preparation and activity of UGT76G1 prepared by pCOLDIII plasmid and Origami2 (DE3) expression strain

The pCOLDIII_UGT76G1 plasmid was transformed into Origami2 (DE3) expression strain (Novagen Origami™2 (DE3) Competent Cells) by heat shock treatment. The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Ampicillin. Suitable colonies were selected and allowed to grow in liquid LBGKP medium containing Ampicillin. Glycerol was added and 400 μL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 30 mL of LBGKP medium (containing Ampicillin). This culture was allowed to shake at 30°C for 8 h. and subsequently used to inoculate 400 mL of production medium containing 60 g/L of "Overnight express instant TB medium" (Novagen, reference 71491-5), 10 g/L of glycerol and 50 mg/L of Kanamycin. The medium was allowed to stir at 12 °C while taking samples to measure the OD (600 nm) and pH. After 68 h, the cells were harvested by centrifugation and frozen. The obtained cell wet weight was 2.53 g.

1.71 g of the obtained pellet was lysed by addition of 6.0 mL of "Bugbuster Master mix" (Novagen, reference 71456) and 1.9 mL of water. The lysate was recovered by centrifugation and kept frozen.

EXAMPLE 13

Determination of activity

Activity tests were performed on a 5 mL scale with 500 µL of thawed lysate for the transformation of Stevioside to Rebaudioside *A* and Rebaudioside *D* to Rebaudioside *M* using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC. The results for the different preparations of UGT76G1 are summarized in the following table.

Example	Plasmid	Expression strain	Transformation activity*	
			Stevioside to Rebaudioside *A*	Rebaudioside *D* to Rebaudioside *M*
7	pET30a+	BL21 (DE3)	29 U mL^{-1}	0.31 U mL^{-1}
8	pET30a+	Tuner (DE3)	33 U mL^{-1}	0.40 U mL^{-1}
9	pMAL	BL21	20 U mL^{-1}	0.15 U mL^{-1}
10	pMAL	ArticExpress	15 U mL^{-1}	0.25 U mL^{-1}
11	pCOLDIII	ArticExpress	15 U mL^{-1}	0.11 U mL^{-1}
12	pCOLDIII	Origami2 (DE3)	37 U mL^{-1}	0.20 U mL^{-1}

* Note

The activities for the transformation of Stevioside and Rebaudioside *M* are mentioned per mL of lysate. 1 U will transform 1 μmol of substrate in 1 hour at 30 °C and pH 7.2

EXAMPLE 14

50 mL scale reaction for the transformation of Rebaudioside *D* to Rebaudioside *M*

5 mL of the lysate of Example 12 was used to transform Rebaudioside *D* to Rebaudioside *M* on a 50 mL scale. The reaction medium consisted of 50 mM Sodium Phosphate buffer pH 7.2, 3 mM of $MgCl_2$, 2.5 mM of UDP-Glucose and 0.5 mM of Rebaudioside *D*. After allowing the reaction to be shaken at 30°C for 90 h. 50 mL of ethanol was added and the resulting mixture was allowed to stir at -20 °C for 1 h. After centrifugation at 5000 g for 10 min. the supernatant was purified via ultrafiltration (Vivaflow MWCO 30000). 78 mL of permeate was obtained and the 9 mL of retentate was diluted with 9 mL of ethanol and resubjected to Ultrafiltration (Vivaflow MWCO 30000). Another 14 mL of filtrate was obtained, which was combined with the first permeate. The combined permeates were concentrated under reduced pressure at 30°C until 32 mL of a clear solution was obtained.

The HPLC trace of the product mixture is shown in FIG. 5. HPLC was carried out on an Agilent 1200 series equipped with a binary pump, auto sampler, and thermostat column compartment. The method was isocratic, with a mobile phase composed of 70% water (0.1% formic acid): 30% acetonitrile. The flow rate was 0.1 μL/min. The column used was Phenomenex Prodigy 5μ ODS (3) 100 A; 250x2mm. The column temperature was maintained at 40 °C. The injection volume was 20-40 μl.

EXAMPLE 15

Preparation of UGT91D2 using pMAL plasmid and BL21 expression strain

After subcloning the synthetic UGT91D2 gene into the pMAL plasmid using NdeI and SalI cloning sites, the pMAL_UGT91D2 plasmid was transformed into BL21 expression strain (New England Biolabs BL21 Competent *E. coli*) by heat shock treatment. The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Ampicillin. Suitable colonies were selected and allowed to grow in liquid LBGKP medium containing Ampicillin). Glycerol was added and 400 µL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 30 mL of LBGKP medium. This culture was allowed to shake at 30°C for 8 h. and subsequently used to inoculate 400 mL of production medium containing 60 g/L of "Overnight express instant TB medium" (Novagen, reference 71491-5), 10 g/L of glycerol and 50 mg/L of Ampicillin. The medium was allowed to stir at 20 °C while taking samples to measure the OD and pH. After 40 h, the cells were harvested by centrifugation and frozen. The obtained cell wet weight is 12.32g.

2.18 g of obtained pellet was lysed by addition of 7.7 mL of "Bugbuster Master Mix" (Novagen, reference 71456) and 3.2 mL of water. The lysate was recovered by centrifugation and used directly for activity testing.

EXAMPLE 16

Preparation of UGT91D2 using pMAL plasmid and ArcticExpress expression strain

The pMAL_UGT91D2 plasmid was transformed into ArcticExpress expression strain (Agilent ArcticExpress competent cells) by heat shock treatment. The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Ampicillin and Geneticin. Suitable colonies were selected and allowed to grow in liquid LBGKP medium containing Ampicillin and Geneticin. Glycerol was added and 400 µL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 30 mL of LBGKP medium (containing Ampicillin and Geneticin). This culture was allowed to shake at 30°C for 8 h. and subsequently used to inoculate 400 mL of production medium containing 60 g/L of "Overnight express instant TB medium" (Novagen, reference 71491-5), 10 g/L of glycerol

and 50 mg/L of Ampicillin. The medium was allowed to stir at 20°C for 16 h. followed by another 50 h. at 12°C while taking samples to measure the OD (600 nm) and pH. The cells were harvested by centrifugation and frozen. The obtained cell wet weight is 15.77 g.

2.57 g of the obtained pellet was lysed by addition of 9.0 mL of "Bugbuster Master Mix" (Novagen, reference 71456) and 3.8 mL of water. The lysate was recovered by centrifugation and used directly for activity testing.

EXAMPLE 17

Preparation of UGT91D2 using pET30a+ plasmid and Tuner (DE3) expression strain

The pET30a+_UGT91D2 plasmid was transformed into Tuner (DE3) expression strain (Novagen Tuner™ (DE3) Competent cells) by heat shock treatment. The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Kanamycin. Suitable colonies were selected and allowed to grow in liquid LBGKP medium (containing Kanamycin). Glycerol was added and 400 µL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 100 mL of LB medium containing 50 mg/L of Kanamycin. This culture allowed to shake at 30°C for 15 h. 6.2 mL of this culture was used to inoculate 500 mL of production medium containing LB. This medium was allowed to stir at 37°C until an OD (600 nm) of 0.9 was obtained after which 500 µL of a 100 mM IPTG solution was added (IPTG concentration in medium is 100 µM) and the medium was allowed to stir at 30 °C for 4 h, the cells were harvested by centrifugation and frozen. The obtained cell wet weight is 4.02 g.

1.92 g of the obtained pellet was lysed by addition of 6.8 mL of "Bugbuster Master mix" (Novagen, reference 71456) and 2.8 mL of water. The lysate was recovered by centrifugation and tested directly for activity.

EXAMPLE 18

Preparation of UGT91D2 using pET30a+ plasmid and ArcticExpress expression strain

The pET30a+_UGT91D2 plasmid was transformed into ArcticExpress (DE3) expression strain (Agilent ArcticExpress competent cells) by heat shock treatment. The obtained cells were grown on LB Agar medium in petri-dishes in the presence of Kanamycin and Geneticin. Suitable colonies were selected and allowed to grow in liquid

LBGKP medium containing of Kanamycin and Geneticin. Glycerol was added and 400 µL aliquots were stored at -20°C and at -80°C.

A storage aliquot was thawed and added to 30 mL of LBGKP medium (containing Kanamycin and Geneticin). This culture was allowed to shake at 30°C for 8 h. and subsequently used to inoculate 400 mL of production medium containing 60 g/L of "Overnight express instant TB medium" (Novagen, reference 71491-5), 10 g/L of glycerol and 50 mg/L of Ampicillin. The medium was allowed to stir at 20°C for 16h. followed by another 50 h. at 12°C while taking samples to measure the OD (600 nm) and pH. After 60 h, the cells were harvested by centrifugation and frozen. The obtained cell wet weight is 16.07 g.

3.24 g of the obtained pellet was lysed by addition of 11.4 mL of "Bugbuster Master Mix" (Novagen, reference 71456) and 4.8 mL of water. The lysate was recovered by centrifugation and used directly for activity testing.

EXAMPLE 19

Determination of activity of in-vivo preparations of UGT91D2

Activity tests were performed at 5 mL scale with 1000 µL of lysate for the transformation of Rubusoside to Stevioside using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC. The results for the different preparations of UGT91D2 are summarized in the following table.

Example	Plasmid	Expression strain	Transformation activity*
			Rubusoside to Stevioside
15	pMAL	BL21	9 mU mL^{-1}
16	pMAL	ArcticExpress	60 mU mL^{-1}
17	pET30a+	Tuner (DE3)	28 mU mL^{-1}
18	pET30a+	ArcticExpress (DE3)	21 mU mL^{-1}

* Note: The activities are mentioned per mL of lysate. 1 U will transform 1 µmol of substrate in 1 hour at 30 °C and pH 7.2

EXAMPLE 20

Other enzymes for Rebaudioside A to Rebaudioside D conversion

The following genes of UDP-glucosyltransferases were identified from public databases, synthesized by DNA2.0 and subsequently subcloned in pET30a+ vector.

Microplate	Position	Gene Name	Internal reference	Conversion RebA to RebD
C908201	A1	gi115454819_NP_001051010.1	S115N01 A1	Active
C908201	G2	gi187373030_ACD03249.1	S115N01 G2	Active
C908201	A7	gi460409128_XP_004249992.1	S115N05 A7	Active
C912666	E1	gi222619587_EEE55719.1	S115N06 E1	Active
C912666	C2	gi297795735_XP_002865752.1	S115N06 C2	Active

The aminoacid sequences are as follows:

SEQ ID 3

>gi|115454819|ref|NP_001051010.1| Os03g0702500 [*Oryza sativa* Japonica Group]

MDDAHSSQSPLHVVIFPWLAFGHLLPCLDLAERLAARGHRVSFVSTPRNLARLPP
VRPELAELVDLVALPLPRVDGLPDGAEATSDVPFDKFELHRKAFDGLAAPFSAFL
DTACAGGKRPDWVLADLMHHWVALASQERGVPCAMILPCSAAVVASSAPPTESS
ADQREAIVRSMGTAAPSFEAKRATEEFATEGASGVSIMTRYSLTLQRSKLVAMRS
CPELEPGAFTILTRFYGKPVVPFGLLPPRPDGARGVSKNGKHDAIMQWLDAQPAK
SVVYVALGSEAPMSADLLRELAHGLDLAGTRFLWAMRKPAGVDADSVLPAGFL
GRTGERGLVTTRWAPQVSILAHAAVCAFLTHCGWGSVVEGLQFGHPLIMLPILGD
QGPNARILEGRKLGVAVPRNDEDGSFDRGGVAGAVRAVVVEEEGKTFFANARKL
QEIVADREREERCIDEFVQHLTSWNELKNNSDGQYP

SEQ ID 4

>gi|187373030|gb|ACD03249.1| UDP-glycosyltransferase [*Avena strigosa*]

MAVKDEQQSPLHILLFPFLAPGHLIPIADMAALFASRGVRCTILTTPVNAAIIRSAV
DRANDAFRGSDCPAIDISVVPFPDVGLPPGVENGNALTSPADRLKFFQAVAELREP
FDRFLADNHPDAVVSDSFFHWSTDAAAEHGVPRLGFLGSSMFAGSCNESTLHNNP
LETAADDPDALVSLPGLPHRVELRRSQMMDPKKRPDHWALLESVNAADQKSFGE
VFNSFHELEPDYVEHYQTTLGRRTWLVGPVALASKDMAGRGSTSARSPDADSCL
RWLDTKQPGSVVYVSFGTLIRFSPAELHELARGLDLSGKNFVWVLGRAGPDSSE

WMPQGFADLITPRGDRGFIIRGWAPQMLILNHRALGGFVTHCGWNSTLESVSAGV
PMVTWPRFADQFQNEKLIVEVLKVGVSIGAKDYGSGIENHDVIRGEVIAESIGKLM
GSSEESDAIQRKAKDLGAEARSAVENGGSSYNDVGRLMDELMARRSSVKVGEDII
PTNDGL

SEQ ID 5

>gi|460409128|ref|XP_004249992.1| PREDICTED: cyanidin-3-O-glucoside 2-O-glucuronosyltransferase-like [*Solanum lycopersicum*]

MSPKLHKELFFHSLYKKTRSNHTMATLKVLMFPFLAYGHISPYLNVAKKLADRGF
LIYFCSTPINLKSTIEKIPEKYADSIHLIELHLPELPQLPPHYHTTNGLPPNLNQVLQK
ALKMSKPNFSKILQNLKPDLVIYDILQRWAKHVANEQNIPAVKLLTSGAAVFSYFF
NVLKKPGVEFPFPGIYLRKIEQVRLSEMMSKSDKEKELEDDDDDDDLLVDGNMQI
MLMSTSRTIEAKYIDFCTALTNWKVVPVGPPVQDLITNDVDDMELIDWLGTKDE
NSTVFVSFGSEYFLSKEDMEEVAFALELSNVNFIWVARFPKGEERNLEDALPKGFL
ERIGERGRVLDKFAPQPRILNHPSTGGFISHCGWNSAMESIDFGVPIIAMPMHLDQP
MNARLIVELGVAVEIVRDDDGKIHRGEIAETLKGVITGKTGEKLRAKVRDISKNLK
TIRDEEMDAAAEELIQLCRNGN

SEQ ID 6

>gi|222619587|gb|EEE55719.1| hypothetical protein OsJ_04191 [*Oryza sativa* Japonica Group]

MHVVMLPWLAFGHILPFAEFAKRVARQGHRVTLFSTPRNTRRLIDVPPSLAGRIR
VVDIPLPRVEHLPEHAEATIDLPSNDLRPYLRRAYDEAFSRELSRLLQETGPSRPD
WVLADYAAYWAPAAASRHGVPCAFLSLFGAAALCFFGPAETLQGRGPYAKTEPA
HLTAVPEYVPFPTTVAFRGNEARELFKPSLIPDESGVSESYRFSQSIEGCQLVAVRS
NQEFEPEWLELLGELYQKPVIPIGMFPPPPPQDVAGHEETLRWLDRQEPNSVVYA
AFGSEVKLTAEQLQRIALGLEASELPFIWAFRAPPDAGDGDGLPGGFKERVNGRG
VVCRGWVPQVKFLAHASVGGFLTHAGWNSIAEGLANGVRLVLLPLMFEQGLNA
RQLAEKKVAVEVARDEDDGSFAANDIVDALRRVMVGEEGDEFGVKVKELAKVF
GDDEVNDRYVRDFLKCLSEYKMQRQG

SEQ ID 7

>gi|297795735|ref|XP_002865752.1| UDP-glucoronosyl/UDP-glucosyl transferase family protein [*Arabidopsis lyrata* subsp. lyrata]

MDDKKEEVMHIAMFPWLAMGHLLPFLRLSKLLAQKGHKISFISTPRNILRLPKLPS
NLSSSITFVSFPLPSISGLPPSSESSMDVPYNKQQSLKAAFDLLQPPLTEFLRLSSPD
WIIYDYASHWLPSIAKELGISKAFFSLFNAATLCFMGPSSSLIEESRSTPEDFTVVPP
WVPFKSTIVFRYHEVSRYVEKTDEDVTGVSDSVRFGYTIDGSDAVFVRSCPEFEPE
WFSLLQDLYRKPVFPIGFLPPVIEDDDDDTTWVRIKEWLDKQRVNSVVYVSLGTE
ASLRREELTELALGLEKSETPFFWVLRNEPQIPDGFEERVKGRGMVHVGWVPQVK
ILSHESVGGFLTHCGWNSVVEGIGFGKVPIFLPVLNEQGLNTRLLQGKGLGVEVLR
DERDGSFGSDSVADSVRLVMIDDAGEEIREKVKLMKGLFGNMDENIRYVDELVG
FMRNDESSQLKEEEEEDDCSDDQSSEVSSETDEKELNLDLKEEKRRISVYKSLSSE
FDDYVANEKMG

The tested plasmids were received in a microtiterplate containing a plasmid as freeze-dried solid in each separate well.

Suspension of plasmids. To each well was added 24 µL of ultra-pure sterile water and the microtiter plate was shaken for 30 minutes at Room Temperature. Subsequently, the plate was incubated at 4°C for 1 hour. The content of each well were further mixed by pipetting up and down. The plasmid quantification was performed by Qubit2.0 analysis using 1 µL of suspension. Determined quantities of plasmids were:

Microtiter plate	Position	Internal reference	[Plasmid] ng/µL
C908201	A1	S115N01 A1	32.8
C908201	G2	S115N01 G2	41.0
C908201	A7	S115N05 A7	56.6
C912666	E1	S115N06 E1	64.0
C912666	C2	S115N06 C2	31.4

Transformation of competent cells with plasmids. Aliquots of chemically competent EC100 cells were taken from freezer at -80°C and stored on ice. The cells were allowed to thaw on ice for 10 minutes. 10 µL of a dilution of above described plasmid solution was added to a sterile microtube of 1.5 mL (in order to transform each cell with 50 pg of DNA) and stored on ice. 100 µL of chemically competent cells was added to each

microtube. After incubation of the chemically competent cells plasmid mixtures on ice for 20 min a thermal shock of 30 seconds at 42°C was performed.

Further incubation was performed on ice for 2 minutes. To each microtube 300 µL of SOC medium was added and the resulting mixture was transferred to a sterile 15 mL tube. After incubate for 1 hour at 37°C while shaking at 135 rpm, the mixture is spread on solid Luria Broth medium containing Kanamycin 50 µg/mL. The petri-dishes are allowed to incubate for 16 hours at 37 °C

Preparation of stock solutions in glycerol and purification of plasmids. To a 50 mL sterile Falcon Tube 10 mL of Luria Broth medium containing 50 µg/mL of Kanamycin was added. The medium was seeded with an isolated colony from the above described Petri dish and the cultures were allowed to incubate for 16 hours at 37°C while shaking at 135 rpm.

To sterile microtube of 1.5 mL containing 300 µL of a 60% sterile glycerol solution, 600 µL of the culture was added. The stock solution was stored at -80°C.

The remainder of the culture was centrifuged at 5,525g for 10 minutes at 10°C and after removal of the supernatant, the pellet was stored on ice. The produced plasmids were purified according to the Qiagen Qiaprep Spin Miniprep kit (ref : 27106) and the plasmid yield was measured at 260 nm. The plasmid solution was stored at 4°C. Plasmid quantities were determined as follows:

Microtiter plate	Position	Internal reference of test	[Plasmid] ng/µL
C908201	A1	S115N01 A1	115.7
C908201	G2	S115N01 G2	120.4
C908201	A7	S115N05 A7	293.8
C912666	E1	S115N06 E1	126.1
C912666	C2	S115N06 C2	98.8

In-vitro Expression of enzymes. 18 µL of plasmid solution (containing approximately 1.5 µg of plasmid) was used for in-vitro expression according to the Promega S30 T7 High-Yield Protein Expression System (ref : L1110) kit. The expression medium was produced as follows:

	S30 Premix Plus	T7 S30 Extract	Total
Trials	30 µL	27 µL	57 µL
reference	20 µL	18 µL	38 µL

The prepared expression medium mix was added to the plasmid solution and the solution was allowed to incubate at 30°C for 3 hours while mixing the mixture every 45 minutes. 5 µL of the mixture was frozen whereas the remainder was used for the catalytic test for the conversion of Rebaudioside A to Rebaudioside D.

Catalytic test for transformation of Rebaudioside A to Rebaudioside D. 430 µL of a reaction mixture containing 0.5 mM Rebaudioside A, 3mM $MgCl_2$, 50 mM phosphate buffer (pH7.2) and 2.5 mM UDP-glucose was added to a 1.5 mL sterile microtube. 52 µL of the enzyme expression medium was added and the resulting mixture was allowed to react at 30 °C for 24 hours. 125 µL samples were taken after 2 hours, 16 hours and 24 hours and added to a 115 µL of 60% methanol and 10 µL of 2 N H_2SO_4. The quenched sample was centrifuged at 18,000 g for 2 minutes at RT. 200 µL was transferred to an HPLC vial and analyzed.

HPLC Analysis The HPLC assay was performed as follows:

Apparatus

Equipment	Supplier	Reference	Lot#
Elite	Hitachi	L-2130	NA
Photodiode Array	Hitachi	L-2455	NA
Corona CAD detector	ESA	70-6186A	CO-2044
Injector 100 µL	Hitachi		NA
Column Synergy 4u Hydro-RP 80A (250 x 4.60 mm)	Phenomenex	00G-4375-E0	588582-12

Instrument conditions

Column Temperature	55°C
Detection	UV 205nm; bw 400 nm CAD detection
Analysis duration	15 min
Injected volume	10 µL
Flow rate	1 mL/min

Mobile phase gradient program

Time (min)	% Water containing 0.04 % acetic acid	% methanol
0	40	60
8	25	75
10	25	75
11	40	60

15	40	60

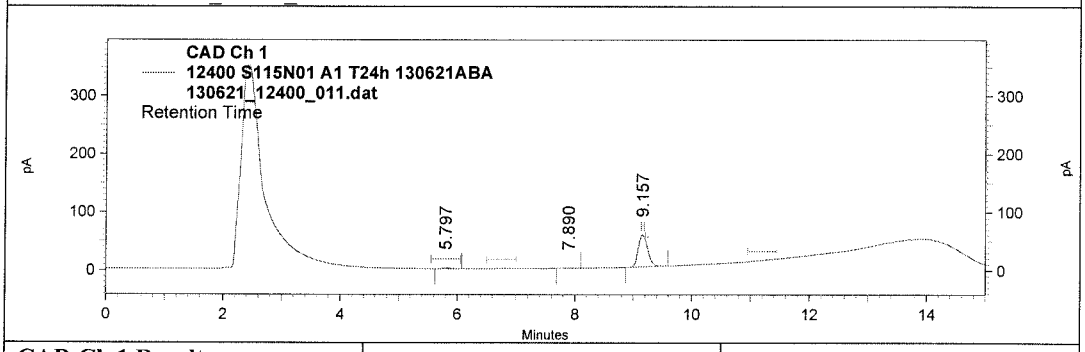
The HPLC assay results are provided below:

Internal reference	Steviol glycoside conversion in reaction mixture (% area)		
	Reb *D*	Reb *UNK*	Reb *A*
S115N01 A1	2.1	ND	96.7
S115N01 G2	0.6	ND	99.4
S115N05 A7	22.4	23.3	46.7
S115N06 E1	0.14	7.0	92.8
S115N06 C2	0.28	3.9	95.8

The enzyme S115N05 A7 had the highest activity for Reb *A* to Reb *D* conversion (ca. 22.4%)

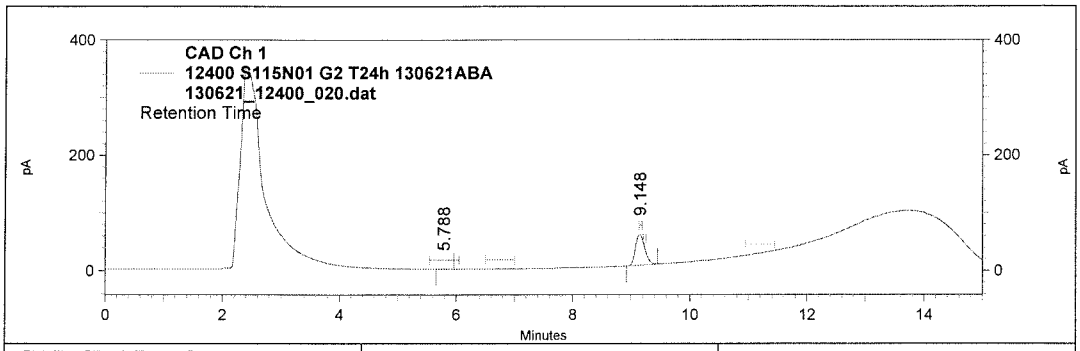
At least three enzymes produced a significant amount of an unknown glycoside (marked as Reb *UNK;* later identified as reb *D2*) along with reb *D*, as seen in Figures 42-46.

Sample : 12400 **S115N01A1** T24h 130621ABA
gi|115454819|ref|NP_001051010.1| Os03g0702500 [*Oryza sativa* Japonica Group]
Filename : 130621_12400_011.dat



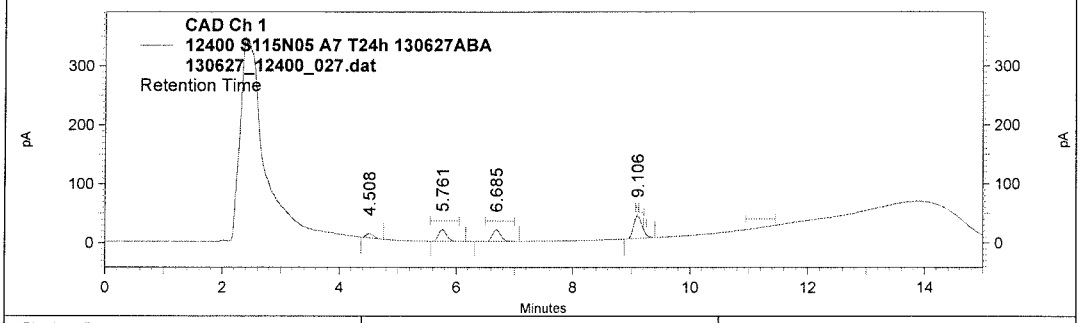
CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside *D*	5.797	13,532,277
Unknown@RT7.890	7.890	7,094,778
Rebaudioside *A*	9.157	613,483,011
Total		634,110,066

Sample : 12400 **S115N01G2** T24h 130621ABA
>gi|187373030|gb|ACD03249.1| UDP-glycosyltransferase [*Avena strigosa*]
Filename : 130621_12400_020.dat



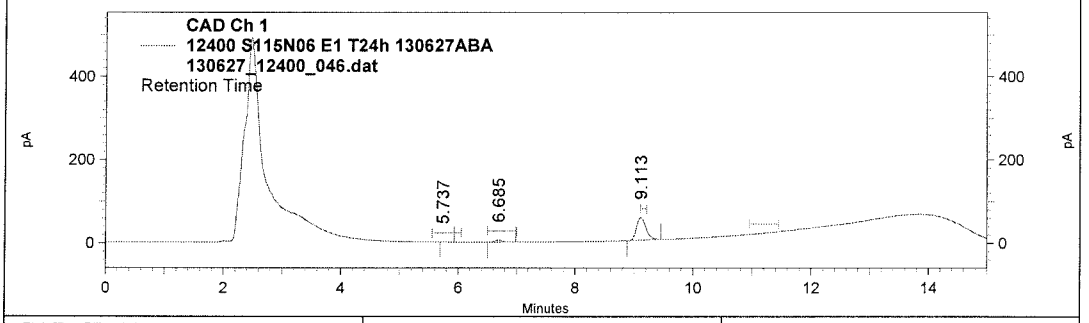
CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside *D*	5.788	3,547,834
Rebaudioside *A*	9.148	585,285,463
Total		588,833,297

Sample : 12400 **S115N05A7** T24h 130627ABA
>gi|460409128|ref|XP_004249992.1| PREDICTED: cyanidin-3-O-glucoside 2-O-glucuronosyltransferase-like [*Solanum lycopersicum*]
Filename : 130627_12400_027.dat



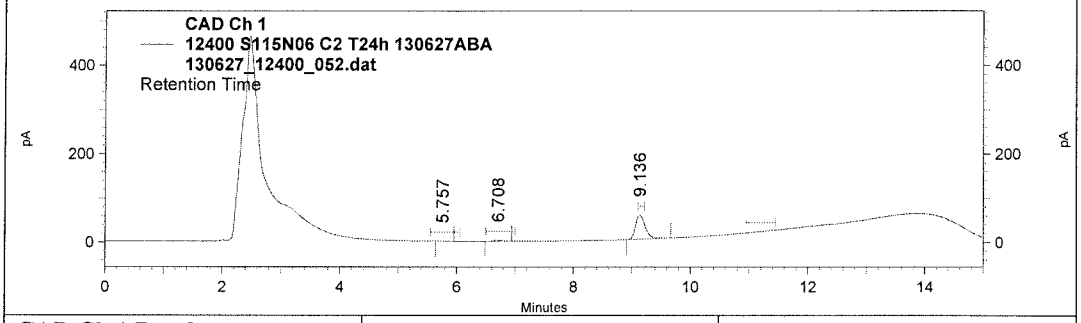
CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Unknown@RT4.508	4.508	64,361,822
Rebaudioside *D*	5.761	191,273,935
Rebaudioside *UNK*	6.685	198,934,644
Rebausioside *A*	9.106	398,115,681
Total		852,686,082

Sample : 12400 **S115N06E1** T24h 130627ABA
>gi|222619587|gb|EEE55719.1| hypothetical protein OsJ_04191 [*Oryza sativa* Japonica Group]
Filename : 130627_12400_046.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside *D*	5.737	964,715
Rebaudioside *UNK*	6.685	46,027,361
Rebausioside *A*	9.113	606,312,523
Total		653,304,599

Sample : 12400 **S115N06C2** T24h 130627ABA
>gi|297795735|ref|XP_002865752.1| UDP-glucoronosyl/UDP-glucosyl transferase family protein [*Arabidopsis lyrata* subsp. lyrata]
Filename : 130627_12400_052.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside *D*	5.757	1,852,407
Rebaudioside *UNK*	6.708	26,033,636
Rebausioside *A*	9.136	633,014,654
Total		660,900,697

EXAMPLE 21

Activity of in-vitro produced EUGT11

EUGT11 gene as was described in the Patent application WO/2013/022989A2 was synthesized by DNA2.0 and subsequently subcloned in pET30a+ vector.

Microplate	Position	GI number	Version	Internal reference	Conversion RebA to RebD
C912666	G4	41469452	AAS07253.1	S115N08 G4	Active

The amino-acid sequence is as follows:

SEQ ID 8

>gi|41469452|gb|AAS07253.1| putative UDP-glucoronosyl and UDP-glucosyl transferase [*Oryza sativa* Japonica Group] EUGT11 enzyme from patent application WO/2013/022989A2

MHVVICPLLAFGHLLPCLDLAQRLACGHRVSFVSTPRNISRLPPVRPSLAPLVSFVA
LPLPRVEGLPNGAESTHNVPHDRPDMVELHLRAFDGLAAPFSEFLGTACADWVM
PTSSAPRQTLSSNIHRNSSRPGTPAPSGRLLCPITPHSNTLERAAEKLVRSSRQNAR
ARSLLAFTSPPLPYRDVFRSLLGLQMGRKQLNIAHETNGRRTGTLPLNLCRWMW

KQRRCGKLRPSDVEFNTSRSNEAISPIGASLVNLQSIQSPNPRAVLPIASSGVRAVFI
GRARTSTPTPPHAKPARSAAPRAHRPPSSVMDSGYSSSYAAAAGMHVVICPWLAF
GHLLPCLDLAQRLASRGHRVSFVSTPRNISRLPPVRPALAPLVAFVALPLPRVEGLP
DGAESTNDVPHDRPDMVELHRRAFDGLAAPFSEFLGTACADWVIVDVFHHWAA
AAALEHKVPCAMMLLGSAHMIASIADRRLERAETESPAAAGQGRPAAAPTFEVA
RMKLIRTKGSSGMSLAERFSLTLSRSSLVVGRSCVEFEPETVPLLSTLRGKPITFLG
LMPPLHEGRREDGEDATVRWLDAQPAKSVVYVALGSEVPLGVEKVHELALGLEL
AGTRFLWALRKPTGVSDADLLPAGFEERTRGRGVVATRWVPQMSILAHAAVGAF
LTHCGWNSTIEGLMFGHPLIMLPIFGDQGPNARLIEAKNAGLQVARNDGDGSFDR
EGVAAAIRAVAVEEESSKVFQAKAKKLQEIVADMACHERYIDGFIQQLRSYKD

The tested plasmid was received in a microtiterplate containing a plasmid as freeze-dried solid in a separate well.

Suspension of plasmid To the well was added 24 µL of ultra-pure sterile water and the microtiter plate was shaken for 30 minutes at Room Temperature. Subsequently, the plate was incubated at 4°C for 1 hour. The content of the well was further mixed by pipetting up and down. The plasmid quantification was performed by Qubit2.0 analysis using 1 µL of suspension. Plasmid quantity was determined as follows:

Microtiter plate	Position	Internal reference of test	[Plasmid] ng/µL
C912666	G4	S115N08 G4	19.2

Transformation of competent cells with plasmid. An aliquot of chemically competent EC100 cells was taken from freezer at -80 °C and stored on ice. The cells were allowed to thaw on ice for 10 minutes. 10 µL of a dilution of above described plasmid solution was added to a sterile microtube of 1.5 mL (in order to transform each cell with 50 pg of DNA) and stored on ice. 100 µL of chemically competent cells was added to the microtube. After incubation of the chemically competent cells/plasmid mixture on ice for 20 min a thermal shock of 30 seconds at 42°C was performed.

Further incubation was performed on ice for 2 minutes. To the microtube 300 µL of SOC medium was added and the resulting mixture was transferred to a sterile 15 mL tube. After incubate for 1 hour at 37°C while shaking at 135 rpm, the mixture is spread on solid Luria Broth medium containing Kanamycin 50 µg/mL. The Petri dish is allowed to incubate for 16 hours at 37°C.

Preparation of stock solutions in glycerol and purification of plasmid. To a 50 mL sterile Falcon Tube 10 mL of Luria Broth medium containing 50 µg/mL of Kanamycin was added. The medium was seeded with an isolated colony from the above described Petri dish and the cultures were allowed to incubate for 16 hours at 37 °C while shaking at 135 rpm.

To sterile microtube of 1.5 mL containing 300 µL of a 60% sterile glycerol solution, 600 µL of the culture was added. The stock solution was stored at -80 °C.

The remainder of the culture was centrifuged at 5,525 g for 10 minutes at 10°C and after removal of the supernatant, the pellet was stored on ice. The produced plasmids were purified according to the Qiagen Qiaprep Spin Miniprep kit (ref : 27106) and the plasmid yield was measured at 260 nm. The plasmid solution was stored at 4°C. Plasmid quantity was determined as follows:

Microtiter plate	Position	Internal reference of test	[Plasmid] ng/µL
C912666	G4	S115N08 G4	38.4

In-vitro Expression of EUGT11. 18 µL of a diluted plasmid solution (containing approximately 1.5 µg of plasmid) was used for in-vitro expression according to the Promega S30 T7 High-Yield Protein Expression System (ref : L1110) kit. The expression medium was produced as follows:

	S30 Premix Plus	T7 S30 Extract	DNA template	Total
Trials	30 µL	27 µL	18 µL (~1.5 µg)	75 µL
reference	20 µL	18 µL	12 µL (~1.0 µg)	50 µL

The prepared expression medium mix was added to the plasmid solution and the solution was allowed to incubate at 30°C for 3 hours while mixing the mixture every 45 minutes. 5 µL of the mixture was frozen whereas the remainder was used for the catalytic test for the conversion of Rebaudioside A to Rebaudioside D.

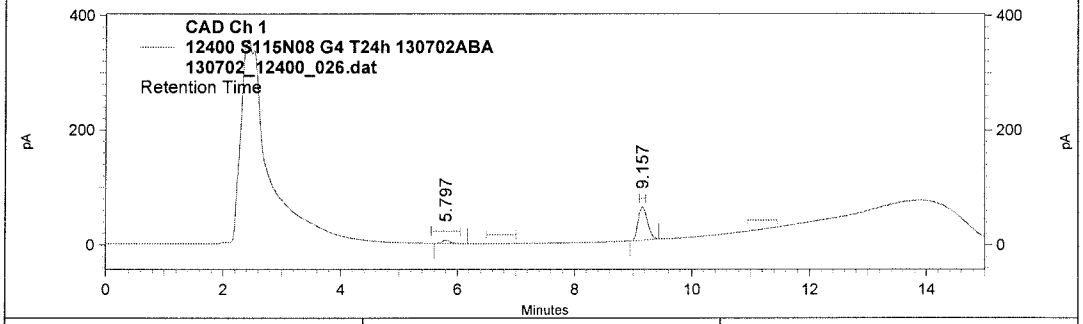
Catalytic test for transformation of Rebaudioside A to Rebaudioside D. 430 µL of a reaction mixture containing 0.5 mM Rebaudioside A, 3mM $MgCl_2$, 50 mM phosphate buffer (pH7.2) and 2.5 mM UDP-glucose was added to a 1,5 mL sterile microtube. 52 µL of the enzyme expression medium was added and the resulting mixture was allowed to react at 30°C for 24 hours. 125 µL samples were taken after 2 hours, 16 hours and 24

hours and added to a 115 µL of 60% methanol and 10 µL of 2 N H_2SO_4. The quenched sample was centrifuged at 18,000 g for 2 minutes at RT. 200 µL was transferred to HPLC vial and analyzed as seen in Figure 47.

HPLC Analysis. The HPLC assay was performed as described in EXAMPLE 20.

The HPLC assay results are provided below:

Sample : 12400 **S115N08G4** T24h 130702CJA
>gi|41469452|gb|AAS07253.1| putative UDP-glucoronosyl and UDP-glucosyl transferase [*Oryza sativa* Japonica Group] (EUGT11 enzyme from Patent application WO/2013/022989A2)
Filename : 130702_12400_026.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside *D*	5.797	54,654,810
Rebaudioside *A*	9.157	633,926,835
Total		688,581,645

EXAMPLE 22

In-vivo production of enzymes

The enzymes described in EXAMPLE 20 were produced *in vivo*.

The pET30A+ vector containing the gene corresponding to the enzyme was introduced in *E. coli* BL21(DE3) by heat shock. The obtained cells were grown in Petri dishes in the presence of Kanamycin and suitable colonies were selected and allowed to grow in liquid LB medium (Erlenmeyer flasks). Glycerol was added to the suspension as cryoprotector and 400 µL aliquots were stored at -20°C and at -80°C.

The storage aliquots of *E. coli* BL21(DE3) containing the pET30A+_UGT plasmids were thawed and added to 30 mL of LBGKP medium (20 g/L Luria Broth Lennox; 50 mM PIPES buffer pH 7.00; 50 mM Phosphate buffer pH 7.00; 2.5 g/L glucose and 50 mg/L of Kanamycine). This culture was allowed to shake at 135 rpm at 30°C for 8hrs.

The production medium contained 60 g/L of overnight express instant TB medium (Novagen), 10 g/L of glycerol and 50 mg/L of Kanamycine. The preculture was added to 400 mL of this medium and the solution was allowed to stir at 20°C while taking samples to measure the OD and pH. The cultures gave significant growth and a good OD was obtained. After 40hrs, the cells were harvested by centrifugation and frozen. The following yields of cell wet weights (CWW) are mentioned below.

GI number	Version	CWW
115454819	NP_001051010.1	9.2 g
187373030	ACD03249.1	7.4 g
460409128	XP_004249992.1	6.8 g
222619587	EEE55719.1	7.5 g
297795735	XP_002865752.1	8.8 g

Lysis was performed by addition of Bugbuster Master mix (Novagen) and the lysate was recovered by centrifugation and used fresh.

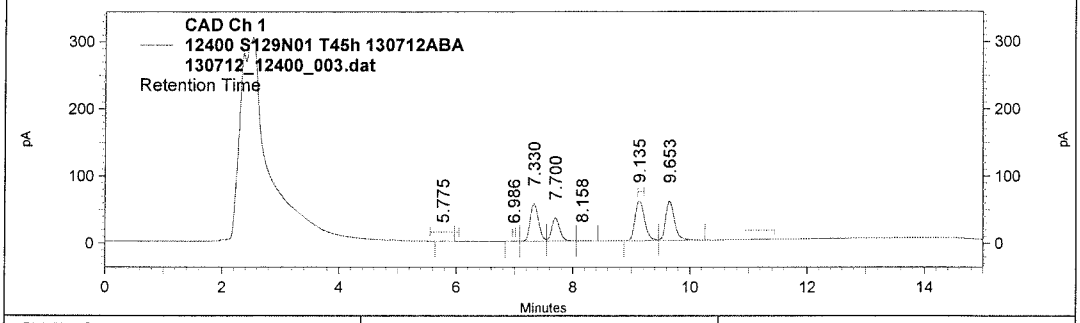
Determination of activity. Activity tests were performed at 5 mL scale with 1,000 µL of thawed lysate for the transformation of Rebaudioside A using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC as seen in Figures 48-52.

HPLC Analysis. The HPLC assay was performed as described in EXAMPLE 20.

The results for the different enzymes are provided below.

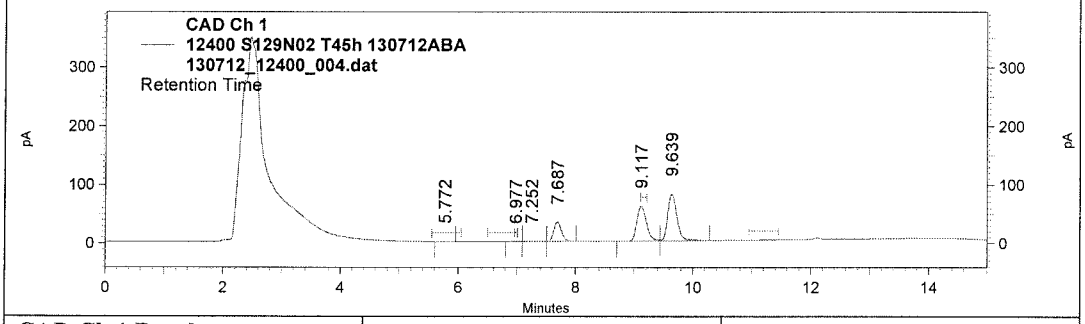
GI Number	Version	Conversion after 45hrs.	Reb *D* selectivity
115454819	NP_001051010.1	1.1%	100%
187373030	ACD03249.1	0.8%	100%
460409128	XP_004249992.1	62.1%	43.6%
222619587	EEE55719.1	2.9%	Reb *D* Not detected
297795735	XP_002865752.1	0.0%	Reb *D* Not detected

SAMPLE : 12400 **S129N01** T45h 130712ABA
> gi115454819 / NP_001051010.1
Filename : 130712_12400_003.dat



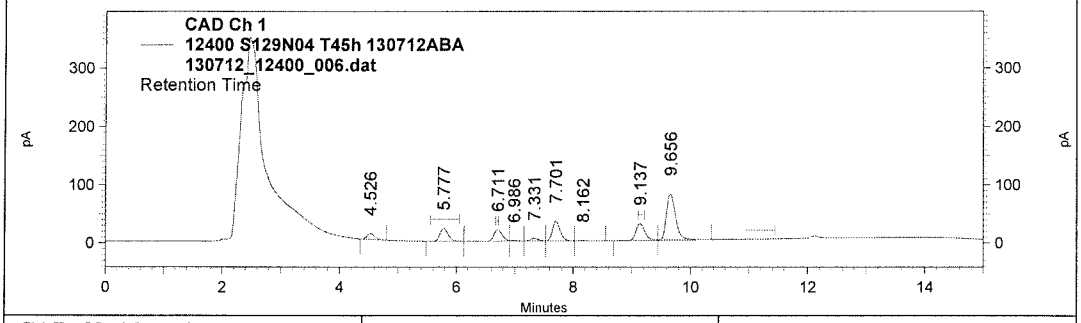
CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside *D*	5.775	3,264,475
Unknown@RT6.986	6.986	4,110,607
Unknown@RT7.330	7.330	564,033,104
Unknown@RT7.700	7.700	328,710,539
Unknown@RT8.158	8.158	6,344,796
Rebaudioside *A*	9.135	673,271,863
Unknown@RT9.653	9.653	616,489,141
Total		2,196,224,525

Sample : 12400 **S129N02** T45h 130712ABA
> gi187373030 / ACD03249.1
Filename : 130712_12400_004.dat



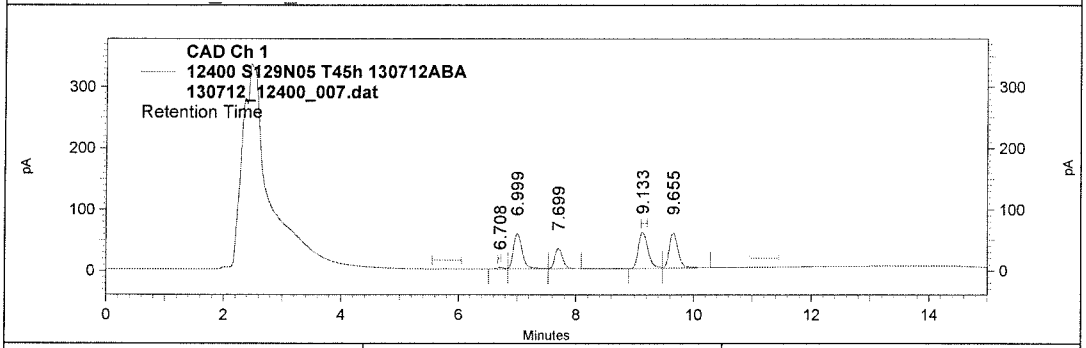
CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside *D*	5.772	1,997,401
Unknown@RT6.977	6.977	3,341,419
Unknown@RT7.252	7.252	10,576,676
Unknown@RT7.687	7.687	298,862,034
Rebaudioside *A*	9.117	675,210,845
Unknown@RT9.639	9.639	874,680,345
Total		1,864,668,720

Sample : 12400 **S129N04** T45h 130712ABA
> gi460409128 / XP_004249992.1
Filename : 130712_12400_006.dat



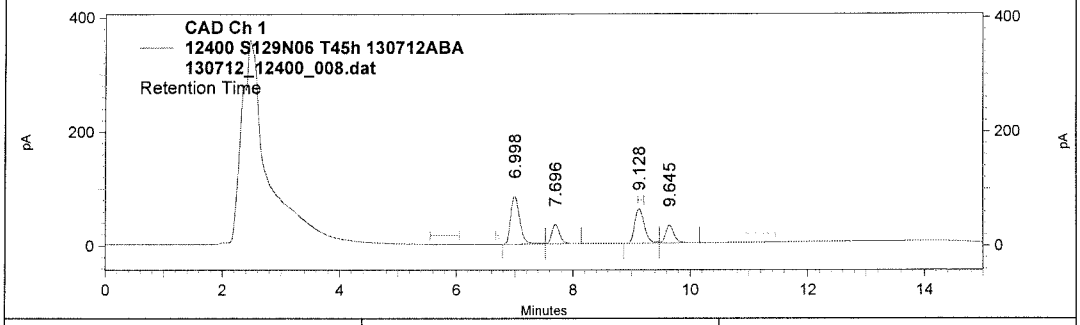
CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Unknown@RT4.526	4.526	89,809,997
Rebaudioside *D*	5.777	217,830,913
Rebaudioside *UNK*	6.711	192,129,243
Unknown@RT6.986	6.986	10,241,417
Unknown@RT7.331	7.331	41,195,765
Unknown@RT7.701	7.701	310,640,254
Unknown@RT8.162	8.162	7,950,609
Rebaudioside *A*	9.137	304,611,760
Unknown@RT9.656	9.656	863,496,704
Total		2,037,906,662

Sample : 12400 **S129N05** T45h 130712ABA
> gi222619587 / EEE55719.1
Filename : 130712_12400_007.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside *UNK*	6.708	20,047,847
Unknown@RT6.999	6.999	598,924,958
Unknown@RT7.699	7.699	303,182,042
Rebaudioside *A*	9.133	672,777,773
Unknown@RT9.655	9.655	606,371,969
Total		2,201,304,589

Sample : 12400 **S129N06** T45h 130712ABA
> gi297795735 / XP_002865752.1
Filename : 130712_12400_008.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Unknown@RT6.998	6.998	920,620,332
Unknown@RT7.696	7.696	314,421,575
Rebaudioside *A*	9.128	688,195,594
Unknown@RT9.645	9.645	308,115,680
Total		2,231,353,181

EXAMPLE 23

Identification of glycosides

The reaction mixtures representing GI No. 460409128, particularly the sample "12400 S115N05A7 T24h 130627ABA" of EXAMPLE 20 (hereinafter S115N05A7), and the sample "12400 S129N04 T45h 130712ABA" of EXAMPLE 22 (hereinafter S129N04) were additionally assayed by LC-MS to identify the unknown glycosides. An Agilent 1200 series HPLC system, equipped with binary pump (G1312B), autosampler (G1367D), thermostatted column compartment (G1316B), DAD detector (G1315C), connected with Agilent 6110A MSD, and interfaced with "LC/MSD Chemstation" software, was used.

Instrument conditions

Column	Phenomenex Kinetex 2.6u C18 100A, 4.6mm x 150mm, 2.6µm
Column Temperature	55°C
Detection	DAD at 210nm bw 360nm MSD (Scan and SIM mode) Mode: ES-API, Negative Polarity Drying gas flow:13.0 L/min Nebulizer pressure:30 psig Drying gas temperature: 270°C
Analysis duration	25 min
Injected volume	2 µL
Flow rate	1 mL/min

Mobile phase gradient program

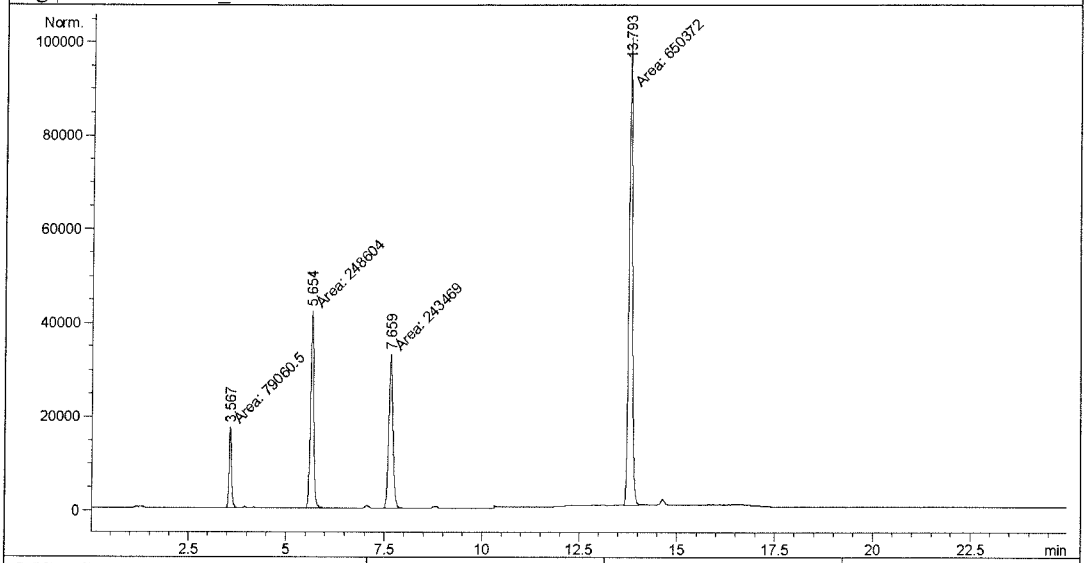
Time (min)	A (%): Formic acid 0.1%	B (%): Acetonitrile
0	75	25
8.5	75	25
10.0	71	29
16.5	70	30

The compound observed on LCMS system at 3.5min, corresponds to compound "Unknown@4.508" in sample "S115N05A7" (EXAMPLE 20), and compound "Unknown@RT4.526" in sample "S129N04" (EXAMPLE 22). The LCMS data suggests that this compound has six glucosidic residues ($C_{56}H_{90}O_{33}$) in its structure, and was found to be an isomer form of reb *M*, namely reb *M2* (see Example 40 for discussion).

Whereas the compound observed on LCMS system at 7.6min, corresponds with compound "reb *UNK*" in sample "S115N05A7" (EXAMPLE 20), and compound "reb *UNK*" in sample "S129N04" (EXAMPLE 22), The LCMS data suggests that "reb *UNK*" has five glucosidic residues ($C_{50}H_{80}O_{28}$) in its structure, and was found to be an isomer form of reb *D*, namely reb D2 (see Example 39 for discussion). The ratio of these compounds and the LCMS chromatograms are provided below and as shown in Figures 53-54.

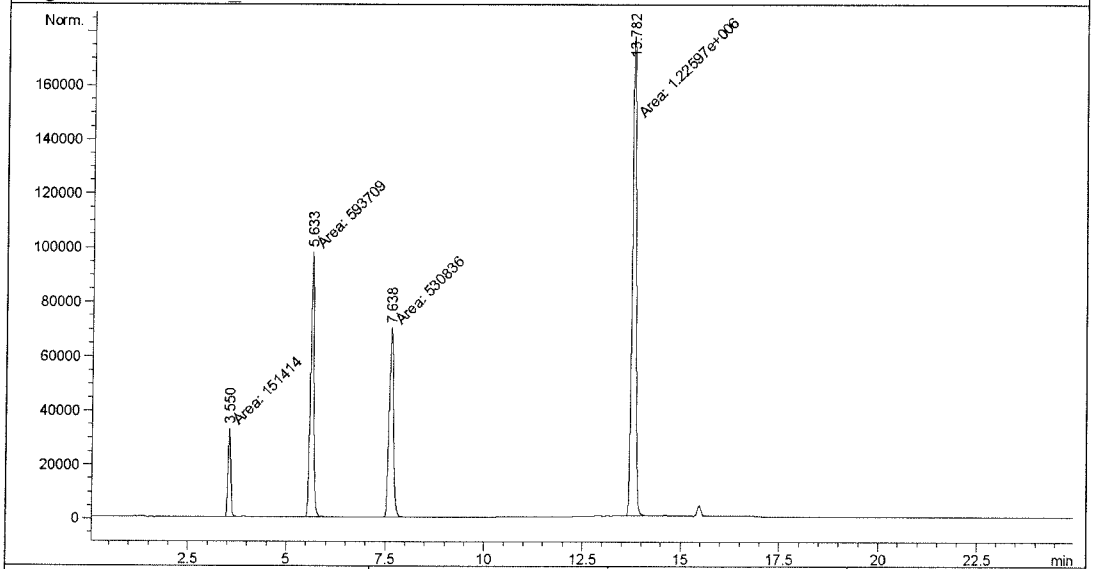
Sample	Steviol glycoside conversion in reaction mixture (% area)			
	Unknown@RT3.5	Reb *D*	Reb *UNK*	Reb *A*
S115N05A7	6.47	20.35	19.93	53.24
S129N04	6.05	23.73	21.22	49.00

Sample : 12400 **S115N05A7** T24h 130627ABA
>gi|460409128/ XP_004249992.1



MSD SIM Results			
Compound	**Retention time**	**MW**	**Integration (area)**
Unknown@RT3.567	3.567	1,291	79,060
Rebaudioside *D*	5.654	1,129	248,604
Rebaudioside *UNK*	7.659	1,129	243,469
Rebausioside *A*	13.793	967	650,372
Total			1,221,505

Sample : 12400 **S129N04** T45h 130712ABA
> gi460409128 / XP_004249992.1



MSD SIM Results			
Compound	**Retention time**	**MW**	**Integration (area)**
Unknown@RT3.550	3.550	1,291	151,414
Rebaudioside *D*	5.633	1,129	593,709
Rebaudioside *UNK*	7.638	1,129	530,836
Rebaudioside *A*	13.782	967	1,225,970

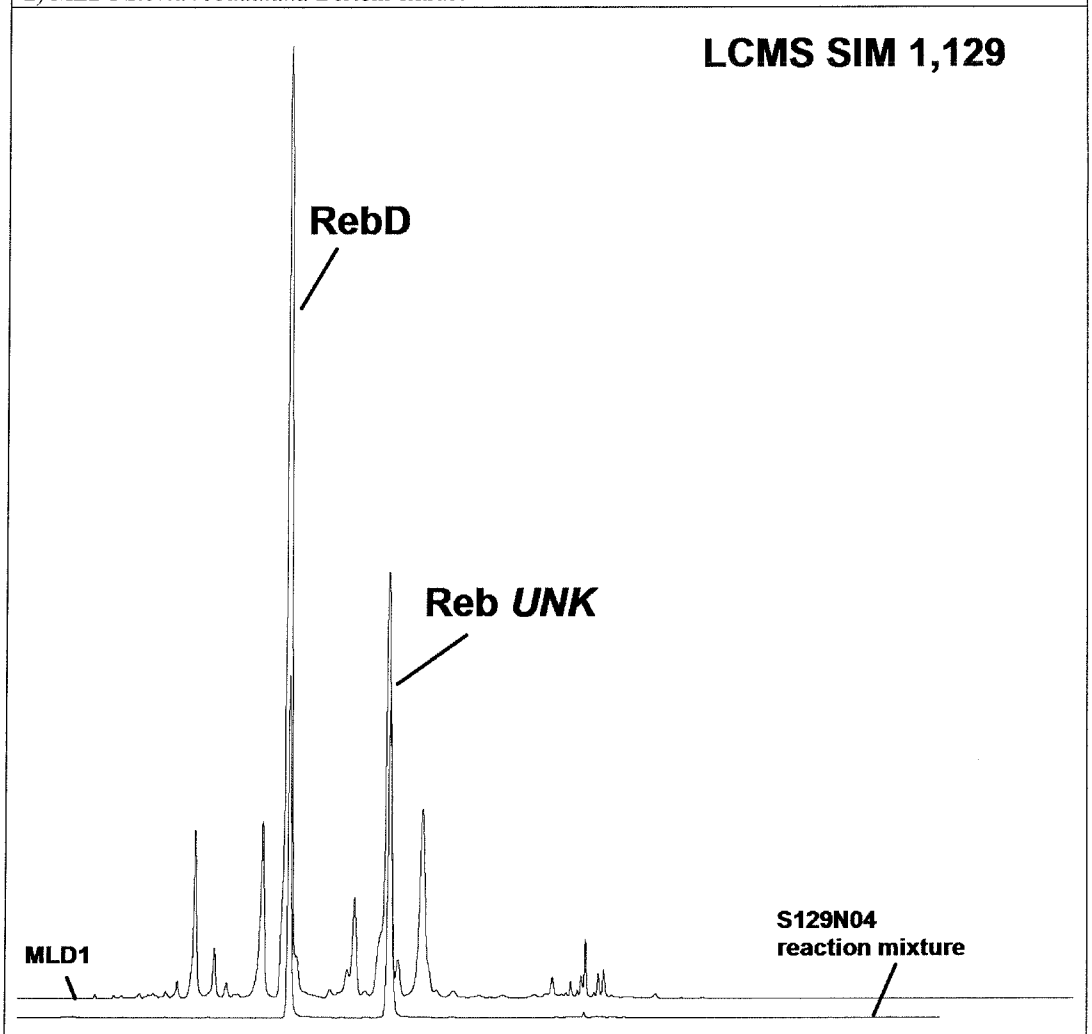
Total			2,501,929

EXAMPLE 24

Identification of glycosides

The reaction mixture representing GI No. 460409128, particularly the sample "12400 S129N04 T45h 130712ABA" of EXAMPLE 22 (hereinafter S129N04) were additionally assayed by LC-MS, as seen in Figures 55-56, along with *Stevia rebaudiana* Bertoni leaf extract "MLD1" produced by PureCircle Sdn Bhd (Malaysia) to determine the occurrence of S129N04 glycosides in nature.

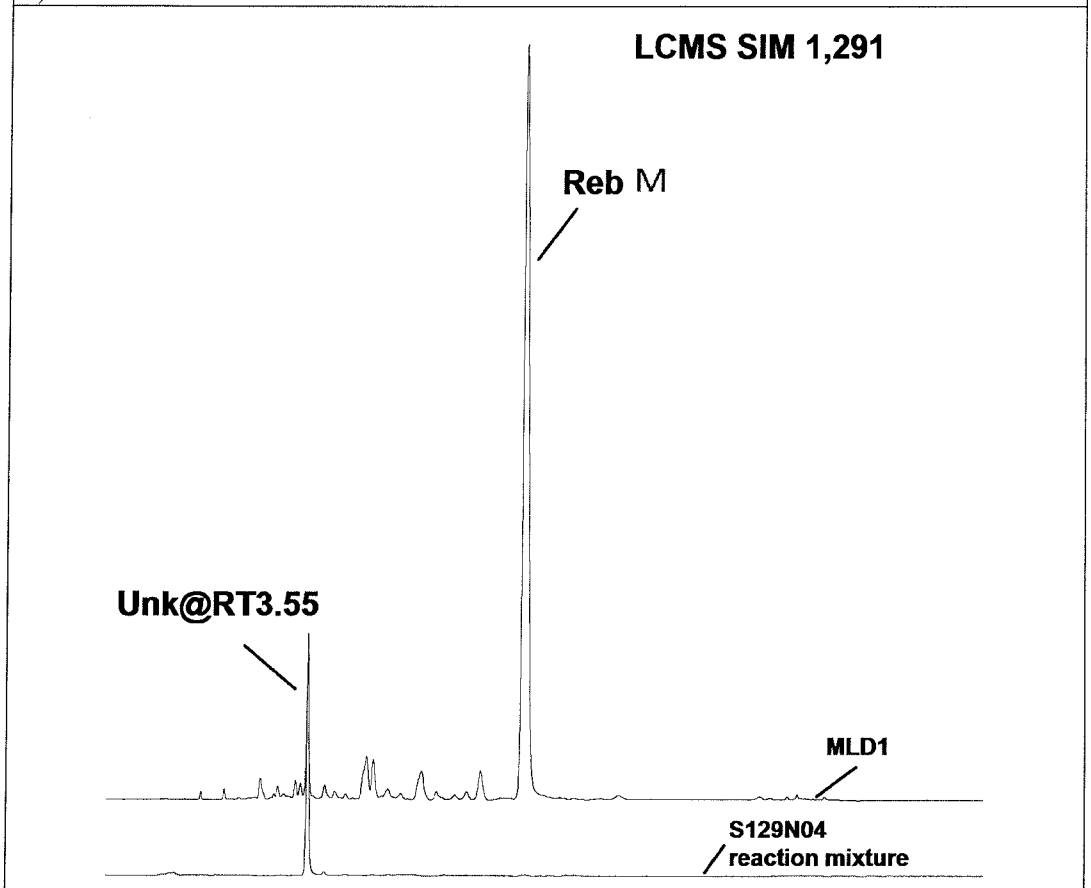
Samples :
1) 12400 **S129N04** T45h 130712ABA
> gi460409128 / XP_004249992.1
2) MLD1 *Stevia rebaudiana* Bertoni extract



MSD SIM 1,129 Results	
Compound	**MW**
Rebaudioside *D*	1,129

Rebaudioside *UNK*	1,129

Samples :
1) 12400 **S129N04** T45h 130712ABA
> gi460409128 / XP_004249992.1
2) MLD1 *Stevia rebaudiana* Bertoni extract



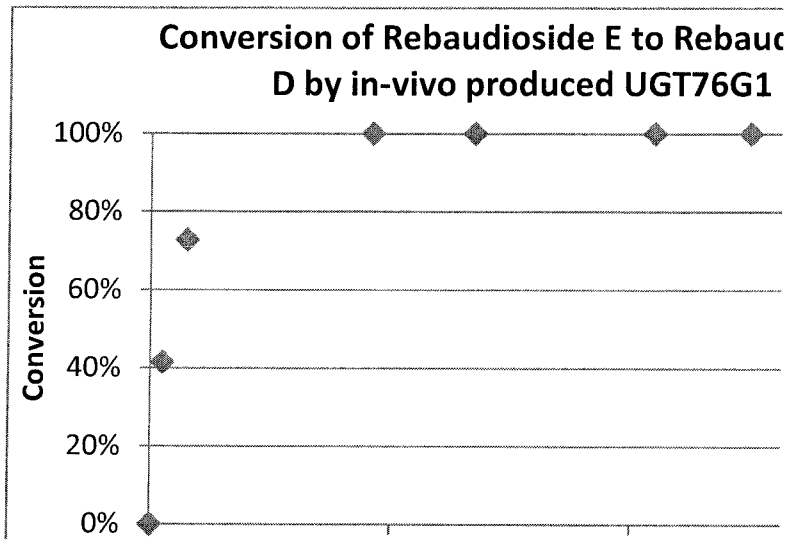
MSD SIM 1,291 Results	
Compound	**MW**
Unknown@RT3.550	1,291
Rebaudioside *M*	1,291

The assay shows that the compound observed on LCMS system at 3.5min, in EXAMPLE 23 ($C_{56}H_{90}O_{33}$; later confirmed as reb *M2*), and the compound observed on LCMS system at 7.6min, in EXAMPLE 23 ($C_{50}H_{80}O_{28;}$ reb *UNK;* later confirmed as reb *D2*) occur in the extract of *Stevia rebaudiana* Bertoni plant.

EXAMPLE 25

Conversion of Rebaudioside *E* to Rebaudioside *D*

The total volume of the reaction was 5.0 mL with the following composition: 100 mM potassium phosphate buffer pH 7.5, 3 mM $MgCl_2$, 2.5 mM UDP-glucose, 0.5 mM Rebaudioside *E* and 500 µL of UGT76G1 thawed lysate (UGT76G1 gene was cloned in pET30a+ vector and expressed in *E. coli* BL21 (DE3)). The reactions were run at 30°C on an orbitary shaker at 135 rpm. For sampling 300 µL of the reaction mixture was quenched with 30 µL of 2N H_2SO_4 and 270 µL of methanol/water (6/4). The samples were immediately centrifuged and kept at 10°C before analysis by HPLC (CAD detection). The following reaction profile was obtained corresponding to a complete conversion of Rebaudioside *E* to Rebaudioside *D* as seen in Figure 57.



EXAMPLE 26

Directed evolution of UGT76G1 for the conversion of Rebaudioside *D* to Rebaudioside *M*

Starting from the amino acid sequence of UGT76G1, as is described in Genbank (AAR06912.1), different mutations at various amino acid positions were identified that could alter the activity of the enzyme for the transformation of Rebaudioside *D* (Reb *D*) to Rebaudioside *M* (Reb *M*). This list of mutations, designed by DNA2.0 ProteinGPS™ strategy, was subsequently used to synthesize 96 variant genes that contained 3, 4 or 5 of these mutations that were codon-optimized for expression in *E. coli*. The genes were subcloned in the pET30a+ plasmid and used for transformation of *E. coli* BL21 (DE3) chemically competent cells. The obtained cells were grown in Petri-dishes on solid LB

medium in the presence of Kanamycin. Suitable colonies were selected and allowed to grow in liquid LB medium in tubes. Glycerol was added to the suspension as cryoprotectant and 400 µL aliquots were stored at -20 °C and at -80 °C.

These storage aliquots of *E. coli* BL21(DE3) containing the pET30a+_UGT76G1var plasmids were thawed and added to LBGKP medium (20 g/L Luria Broth Lennox; 50 mM PIPES buffer pH 7.00; 50 mM Phosphate buffer pH 7.00; 2.5 g/L glucose and 50 mg/L of Kanamycine). This culture was allowed to shake in a 96 microtiter plate at 135 rpm at 30°C for 8 h.

3.95 mL of production medium containing 60 g/L of Overnight Express™ Instant TB medium (Novagen®), 10 g/L of glycerol and 50 mg/L of Kanamycin was inoculated with 50 µL of above described culture. In a 48 deepwell plate the resulting culture was allowed to stir at 20°C. The cultures gave significant growth and a good OD (600 nm; 1 cm) was obtained. After 44 h, the cells were harvested by centrifugation and frozen.

Lysis was performed by addition of Bugbuster® Master mix (Novagen®) to the thawed cells and the lysate was recovered by centrifugation. Activity tests were performed with 100 µL of fresh lysate that was added to a solution of Rebaudioside *D* (final concentration 0.5 mM), $MgCl_2$ (final concentration 3 mM) and UDP-Glucose (final concentration 2.5 mM) in 50 mM phosphate buffer pH 7.2.

The reaction was allowed to run at 30°C and samples were taken after 2, 4, 7 and 24 h. to determine conversion and initial rate by HPLC (CAD detection) using the analytical method that was described above for the transformation of Rebaudioside *D* to Rebaudioside *M*. The results are depicted in the following table.

Clone	Mutations*	conversion Reb D to Reb M after 24 h (%)	initial rate (Reb M area/min)
UGT76G1var1	E224A_F314S_R334K	51.8	5.5E+07
UGT76G1var2	S274G_T284I_L379G	49.3	4.7E+07
UGT76G1var3	I295T_S357C_V366I	9.6	1.6E+06
UGT76G1var4	E224D_E231A_F265I	14.7	8.6E+06
UGT76G1var5	F22Y_I373L_P382M	3.5	2.3E+06
UGT76G1var6	Q266S_S357N_I373L	0.5	1.8E+06
UGT76G1var7	F22L_I43V_A239V	0.2	-6.0E+04
UGT76G1var8	E224A_Q266S_Q342E	0.5	2.3E+04
UGT76G1var9	E231A_D301N_G348P	52.0	4.9E+07

Clone	Mutations*	conversion Reb D to Reb M after 24 h (%)	initial rate (Reb M area/min)
UGT76G1var10	A33G_L246F_Q342E	0.3	-7.7E+02
UGT76G1var11	F22L_A33G_V310I	0.4	3.8E+04
UGT76G1var12	L243P_K303G_A352G	0.5	8.7E+04
UGT76G1var13	L243A_S357C_A385T	0.2	-3.3E+04
UGT76G1var14	A239I_F265I_V396F	5.3	1.5E+06
UGT76G1var15	F41L_L246F_Q425E	5.6	1.5E+06
UGT76G1var16	F265I_P272A_I335V	18.6	5.8E+06
UGT76G1var17	F265L_Q266E_Q342K	0.7	7.2E+05
UGT76G1var18	L243P_S274G_N409R	1.9	5.0E+05
UGT76G1var19	E224D_E229A_Q432E	10.5	5.5E+06
UGT76G1var20	S375M_K393G_Y397E	1.8	1.9E+06
UGT76G1var21	A239V_V300A_K303G	41.9	3.3E+07
UGT76G1var22	E231A_V310I_R334K	34.4	2.4E+07
UGT76G1var23	T263S_G348P_A352G	47.8	4.1E+07
UGT76G1var24	A239I_P272A_Q425E	31.0	2.1E+07
UGT76G1var25	T284L_Q342K_Y397Q	0.9	6.3E+04
UGT76G1var26	S241I_F265L_F377C	1.8	7.5E+05
UGT76G1var27	A239I_L379A_V394I	29.0	1.5E+07
UGT76G1var28	L243A_S274G_P382M	6.1	2.4E+06
UGT76G1var29	F22Y_V279I_N409R	41.0	2.9E+07
UGT76G1var30	I43V_E224A_S241I	13.6	5.6E+06
UGT76G1var31	E224D_L243P_V300A	0.4	2.4E+05
UGT76G1var32	A239V_L243A_S375M	0.0	-4.4E+04
UGT76G1var33	A33G_R334H_Y397Q	1.0	7.5E+06
UGT76G1var34	I43V_T284I_I295T	3.4	1.5E+06
UGT76G1var35	T284L_F314S_S357N	0.5	1.8E+05
UGT76G1var36	F265L_L379A_V396F	20.0	8.8E+06
UGT76G1var37	E229A_L379G_I407V	39.1	2.8E+07
UGT76G1var38	F41L_I295M_F377C	8.2	3.7E+06
UGT76G1var39	F22Y_F41L_V366I	7.2	3.3E+06
UGT76G1var40	T263S_Q266E_S375R	47.6	3.3E+07
UGT76G1var41	L246F_A385T_K393G	0.8	1.4E+06
UGT76G1var42	T263S_Q266S_R334H	34.6	2.2E+07
UGT76G1var43	S241I_P272A_V279I	19.9	9.4E+06
UGT76G1var44	I335V_S375R_I407V	35.3	2.3E+07
UGT76G1var45	V279I_D301N_S389E	38.6	2.3E+07
UGT76G1var46	F22L_Q266E_I295M	0.6	9.8E+05
UGT76G1var47	E229A_T284I_S389E	4.8	2.7E+06
UGT76G1var48	V394I_Y397E_Q432E	47.6	3.8E+07
UGT76G1var49	F41L_Q266E_T284I_Y397Q	2.6	1.1E+06
UGT76G1var50	F22Y_V310I_S375M_F377C	1.9	7.9E+05

Clone	Mutations*	conversion Reb D to Reb M after 24 h (%)	initial rate (Reb M area/min)
UGT76G1var51	K303G_S357C_S389E_V396F	18.7	9.5E+06
UGT76G1var52	D301N_I373L_F377C_I407V	12.9	4.6E+06
UGT76G1var53	R334K_A352G_P382M_S389E	9.3	4.1E+06
UGT76G1var54	E229A_T284L_R334K_Q342E	0.7	4.3E+05
UGT76G1var55	I295M_Q342E_V366I_N409R	1.0	2.2E+05
UGT76G1var56	L246F_A352G_S357N_Q432E	0.4	4.1E+04
UGT76G1var57	S241I_T263S_L379G_A385T	0.8	1.5E+05
UGT76G1var58	S357C_S375M_N409R_Q425E	7.5	2.2E+06
UGT76G1var59	I335V_K393G_V394I_Y397Q	33.0	2.7E+07
UGT76G1var60	E231A_L243A_V279I_S357N	0.5	9.5E+04
UGT76G1var61	I43V_F265I_Q266S_L379A	6.4	2.0E+06
UGT76G1var62	L243P_P272A_V394I_V396F	0.1	3.4E+04
UGT76G1var63	F314S_R334H_Q342K_L379G	3.4	1.2E+06
UGT76G1var64	F22L_A239I_R334H_I407V	0.3	3.1E+04
UGT76G1var65	A33G_A239V_P382M_Q425E	1.2	3.3E+05
UGT76G1var66	F265L_V310I_V366I_A385T	0.8	3.7E+05
UGT76G1var67	E224D_F314S_S375R_Y397E	-2.1	-5.6E+05
UGT76G1var68	Q342K_G348P_I373L_Y397E	-1.4	-1.1E+05
UGT76G1var69	S274G_I295T_I335V_L379A	24.7	8.3E+06
UGT76G1var70	E224A_I295T_V300A_G348P	24.0	8.4E+06
UGT76G1var71	I295M_V300A_K393G_Q432E	42.9	2.1E+07
UGT76G1var72	T284L_D301N_K303G_S375R	19.2	9.1E+06
UGT76G1var73	F22Y_D301N_R334H_Q342E_V396F	0.8	8.7E+05
UGT76G1var74	I295T_I373L_S375R_Y397Q_Q432E	0.6	9.6E+04
UGT76G1var75	F41L_A239I_Q266S_S375M_P382M	0.8	-1.3E+05
UGT76G1var76	F22Y_A239I_L246F_I295M_R334K	2.6	7.2E+05
UGT76G1var77	A239V_F265I_I295T_D301N_K393G	1.9	4.4E+05
UGT76G1var78	V279I_V300A_V310I_I335V_S357C	3.2	8.2E+05
UGT76G1var79	E224D_T284I_V366I_I373L_K393G	8.5	3.8E+06
UGT76G1var80	L243P_L379A_S389E_Q425E_Q432E	1.0	2.1E+05
UGT76G1var81	A33G_T263S_S274G_V279I_Y397E	15.0	6.5E+06
UGT76G1var82	E224D_L243A_F265L_R334H_A352G	1.1	2.5E+05
UGT76G1var83	I43V_Q342E_S357N_S375R_L379G	0.5	4.3E+04
UGT76G1var84	F22L_Q266S_F314S_A352G_S357C	1.2	2.3E+05
UGT76G1var85	T284L_G348P_F377C_P382M_N409R	1.8	4.0E+05
UGT76G1var86	E224A_T284L_V396F_Y397E_I407V	1.6	3.8E+05
UGT76G1var87	S241I_L243A_V300A_F314S_N409R	35.7	2.1E+07
UGT76G1var88	A239V_T284I_V310I_Q342K_L379A	1.6	3.8E+05
UGT76G1var89	F41L_E229A_E231A_F265L_P272A	1.2	2.1E+05
UGT76G1var90	E231A_S241I_S274G_Y397Q_Q425E	34.5	1.9E+07
UGT76G1var91	E224A_L246F_T263S_F265I_Q342K	1.2	2.3E+05

Clone	Mutations*	conversion Reb D to Reb M after 24 h (%)	initial rate (Reb M area/min)
UGT76G1var92	K303G_S357N_V366I_V394I_I407V	1.6	3.6E+05
UGT76G1var93	I43V_Q266E_S375M_S389E_V394I	1.8	4.5E+05
UGT76G1var94	Q266E_P272A_R334K_G348P_L379G	72.0	7.9E+07
UGT76G1var95	A33G_I295M_K303G_I335V_A385T	-1.3	-1.7E+05
UGT76G1var96	F22L_E229A_L243P_F377C_A385T	1.2	2.7E+05

*Mutations are noted as follows: original amino acid-position-new amino acid: For example the mutation of an alanine at position 33 to a glycine is noted as A33G.

EXAMPLE 27

In-vivo production of UGTSL2

SEQ ID 9

UGTSL2 (GI_460410132 / XP_004250485.1) amino acid sequence:

MATNLRVLMFPWLAYGHISPFLNIAKQLADRGFLIYLCSTRINLESIIKKIPEKYAD
SIHLIELQLPELPELPPHYHTTNGLPPHLNPTLHKALKMSKPNFSRILQNLKPDLLIY
DVLQPWAEHVANEQNIPAGKLLTSCAAVFSYFFSFRKNPGVEFPFPAIHLPEVEKV
KIREILAKEPEEGGRLDEGNKQMMLMCTSRTIEAKYIDYCTELCNWKVVPVGPPF
QDLITNDADNKELIDWLGTKHENSTVFVSFGSEYFLSKEDMEEVAFALELSNVNFI
WVARFPKGEERNLEDALPKGFLERIGERGRVLDKFAPQPRILNHPSTGGFISHCGW
NSAMESIDFGVPIIAMPIHNDQPINAKLMVELGVAVEIVRDDDGKIHRGEIAETLKS
VVTGETGEILRAKVREISKNLKSIRDEEMDAVAEELIQLCRNSNKSK

The pET30A+ vector containing the UGTSL2 gene was introduced in *E. coli* Bl21(DE3) by heat shock. The obtained cells were grown in petri-dishes in the presence of Kanamycin and suitable colonies were selected and allowed to grow in liquid LB medium (erlenmeyer flasks). Glycerol was added to the suspension as cryoprotecteur and 400 µL aliquots were stored at -20°C and at -80°C.

The storage aliquots of *E. coli* BL21(DE3) containing the pET30A+_UGTSL2 plasmids were thawed and added to 30 mL of LBGKP medium (20 g/L Luria Broth Lennox; 50 mM PIPES buffer pH 7.00; 50 mM Phosphate buffer pH 7.00; 2.5 g/L glucose and 50 mg/L of Kanamycin). This culture was allowed to shake at 135 rpm at 30°C for 8 h.

The production medium contained 60 g/L of overnight express instant TB medium (Novagen), 10 g/L of glycerol and 50 mg/L of Kanamycin. The preculture was added to 200 mL of this medium and the solution was allowed to stir at 20°C while taking samples to measure the OD and pH. The culture gave significant growth and a good OD was obtained. After 40 h, the cells were harvested by centrifugation and frozen to obtain 6.22 g of cell wet weight.

Lysis was performed on 1.4 g of cells by addition of Bugbuster Master mix (Novagen) and the lysate was recovered by centrifugation and used fresh.

EXAMPLE 28

Determination of activity for Stevioside to Rebaudioside *E* conversion with UGTSL and UGTSL2

UGTSL was prepared according to EXAMPLE 22, and UGTSL2 was prepared according to EXAMPLE 27.

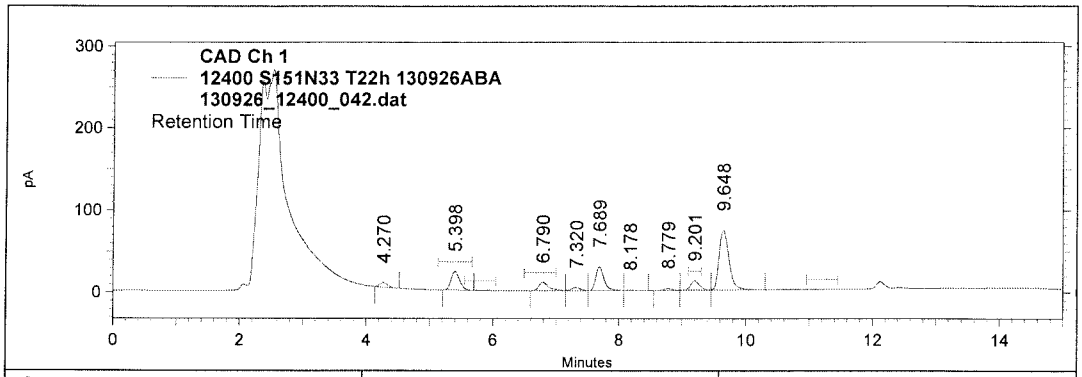
Activity tests were performed at 3 mL scale with 600 µL of lysate for the transformation of Stevioside using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC. HPLC Analysis as shown in Figures 58-59. The HPLC assay was performed as described in EXAMPLE 20.

The results for the different enzymes and the corresponding chromatograms are provided below.

Enzyme internal reference	GI Number	Version	Stevioside conv.[1] (reaction time)	Rebaudioside *E* formation[1]
UGTSL	460409128	XP_004249992.1	74% (22 h.)	46%
UGTSL2	460410132	XP_004250485.1	77% (2 h.)	50%

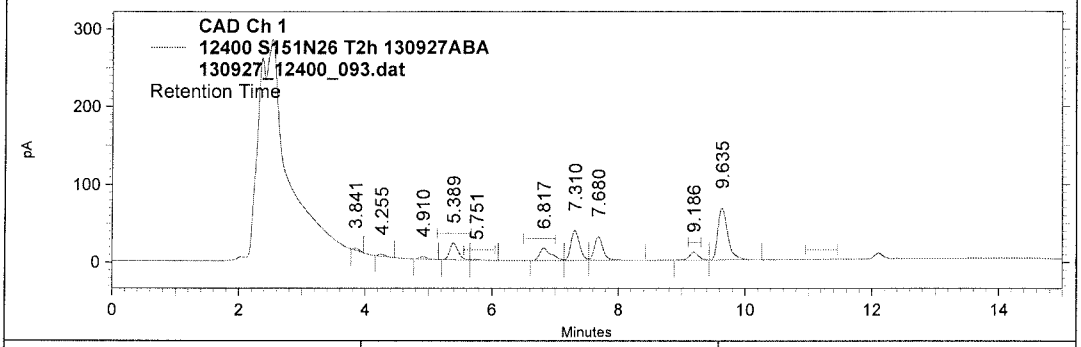
Note: [1]Based on initial concentration of Stevioside

SAMPLE : 12400 S151N33 T22h 130926ABA
Gene references: UGTSL (XP_004249992.1)
Filename : 130926_12400_042.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Unknown @RT4.27	4.270	45,634,692
Rebaudioside *E*	5.398	215,079,800
Unknown @RT6.79	6.790	11,0326,212
Unknown @RT7.32	7.320	33,855,010
Unknown @RT7.69	7.689	271,186,269
Unknown @RT8.18	8.178	6,003,490
Unknown @RT8.78	8.779	20,739,231
Stevioside	9.201	114,734,548
Unknown @RT9.65	9.648	779,225,521
Total		1,596,784,773

SAMPLE : 12400 S151N26 T2h 130927ABA
Gene references: UGTSL2 (XP_004250485.1)
Filename : 130927_12400_093.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Unknown @RT3.84	3.841	16,182,482
Unknown @RT4.25	4.255	20,078,830
Unknown @RT4.91	4.910	27,630,795
Rebaudioside *E*	5.389	203,768,956
Unknown @RT5.75	5.751	8,018,638
Unknown @RT6.82	6.817	200,959,602
Unknown @RT7.31	7.310	370188401
Unknown @RT7.68	7.680	294,963,186
Stevioside	9.186	101,729,292
Unknown @RT9.63	9.635	727,903,255
Total		1,971,423,437

EXAMPLE 29

Determination of activity for Rubusoside to Rebaudioside E conversion with UGTSL and UGTSL2

UGTSL was prepared according to EXAMPLE 22, and UGTSL2 was prepared according to EXAMPLE 27.

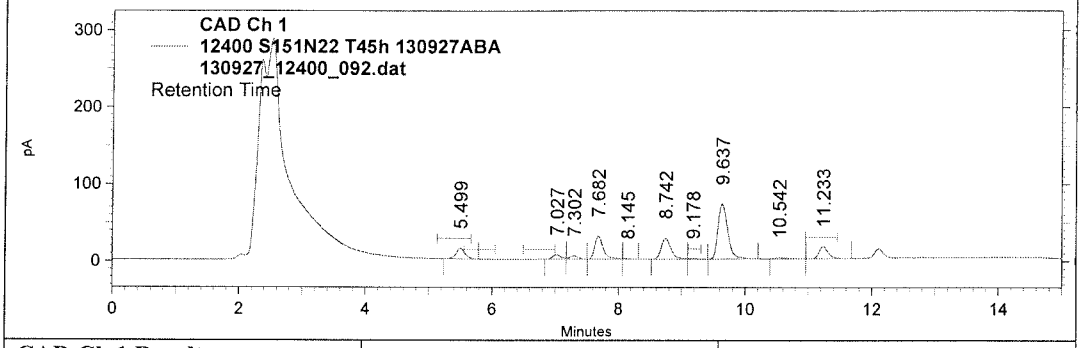
Activity tests were performed at 3 mL scale with 600 µL of lysate for the transformation of Rubusoside using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC as shown in Figures 60-61. The HPLC assay was performed as described in EXAMPLE 20.

The results for the different enzymes and the corresponding chromatograms are provided below.

Enzyme internal reference	GI Number	Version	Rubusoside conv.[1] (reactiontime)	Rebaudioside E formation[1]
UGTSL	460409128	XP_004249992.1	70% (45 h.)	27%
UGTSL2	460410132	XP_004250485.1	80% (2 h.)	55%

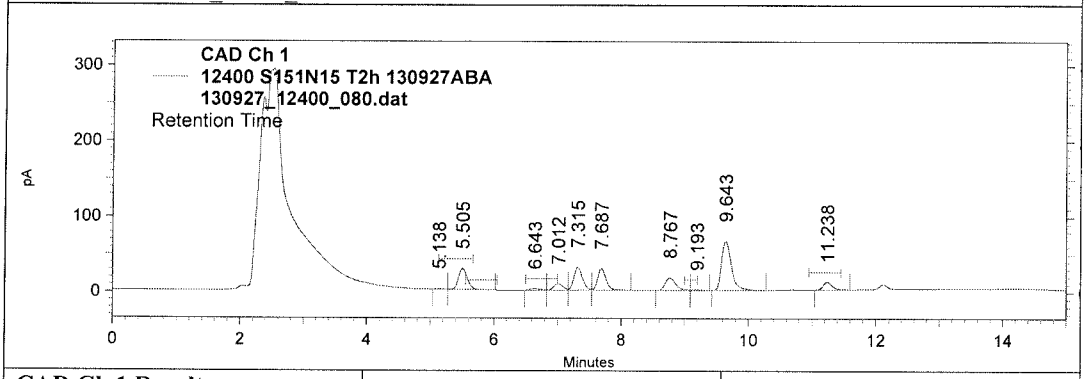
Note: [1]Based on initial concentration of Rubusoside

SAMPLE : 12400 S151N22 T45h 130927ABA
Gene references: UGTSL (XP_004249992.1)
Filename : 130927_12400_092.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside *E*	5.499	135,984,743
Unknown @RT7.03	7.027	54,448,761
Unknown @RT7.30	7.302	41,308,528
Unknown @RT7.68	7.682	283,852,603
Unknown @RT8.14	8.145	5,484,731
Unknown @RT8.74	8.742	290,946,055
Stevioside	9.178	8,774,098
Unknown @RT9.64	9.637	761,299,117
Unknown @RT10.54	10.542	18,276,224
Rubusoside	11.233	155,492,389
Total		1,755,867,249

SAMPLE : 12400 S151N15 T2h 130927ABA
Gene references: UGTSL2 (XP_004250485.1)
Filename : 130927_12400_080.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Unknown @RT5.14	5.138	5,555,472
Rebaudioside *E*	5.505	278,529,547
Unknown @RT6.64	6.643	23,812,633
Unknown @RT7.01	7.012	84,543,823
Unknown @RT7.31	7.315	283,724,517
Unknown @RT7.69	7.687	264,400,008
Unknown @RT8.78	8.767	188,634,123
Stevioside	9.193	9,365,107
Unknown @RT9.64	9.643	700,878,865
Rubusoside	11.238	102,484,386
Totals		1,941,928,481

EXAMPLE 30

Determination of activity for Rebaudioside *A* to Rebaudioside *D* conversion with UGTSL2

UGTSL2 was prepared according to EXAMPLE 27.

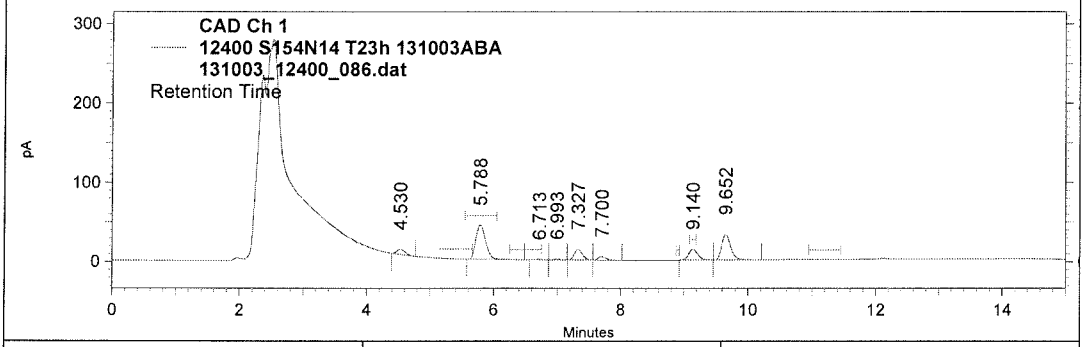
Activity tests were performed at 3 mL scale with 60 µL of lysate for the transformation of Rebaudioside *A* using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC as shown in Figure 62. The HPLC assay was performed as described in EXAMPLE 20.

The result after 23 h. of reaction and the corresponding chromatogram is provided below.

Enzyme internal reference	GI Number	Version	Rebaudioside *A* conv.[1] (reaction time)	Rebaudioside *D* formation[1]
UGTSL2	460410132	XP_004250485.1	78% (23 h.)	75%

Note: [1]Based on initial concentration of Rebaudioside *A*

SAMPLE : 12400 S154N14 T23h 131003ABA
Gene references: UGTSL2 (XP_004250485.1)
Filename : 131003_12400_086.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Unknown @RT4.53	4.530	55,894,278
Rebaudioside *D*	5.788	461,768,318
Unknown @RT6.71	6.713	7,942,480
Unknown @RT6.99	6.993	11,192,896
Unknown @RT7.33	7.327	120,255,606
Unknown @RT7.70	7.700	38,994,186
Rebaudioside *A*	9.140	137,037,966
Unknown @RT9.65	9.652	314,468,535
Total		1,147,554,265

EXAMPLE 31

Identification of glycosides

The reaction mixtures prepared according to EXAMPLE 30 and incubated for 45hrs was analyzed by LC-MS, along with *Stevia rebaudiana* Bertoni leaf extract "MLD1" produced by PureCircle Sdn Bhd (Malaysia), to determine the occurrence of formed glycosides in nature.

An Agilent 1200 series HPLC system, equipped with binary pump (G1312B), autosampler (G1367D), thermostatted column compartment (G1316B), DAD detector (G1315C), connected with Agilent 6110A MSD, and interfaced with "LC/MSD Chemstation" software, was used, and the chromatogram is shown in Figure 63.

Instrument conditions

Column	Phenomenex Prodigy 3u C18 100A, 4.6mm x 250mm, 3μm
Column Temperature	55°C
Detection	DAD at 210nm bw 360nm MSD (Scan and SIM mode) Mode: ES-API, Negative Polarity Drying gas flow:13.0 L/min Nebulizer pressure:30 psig Drying gas temperature: 270°C
Analysis duration	75 min
Injected volume	10 μL
Flow rate	0.5 mL/min

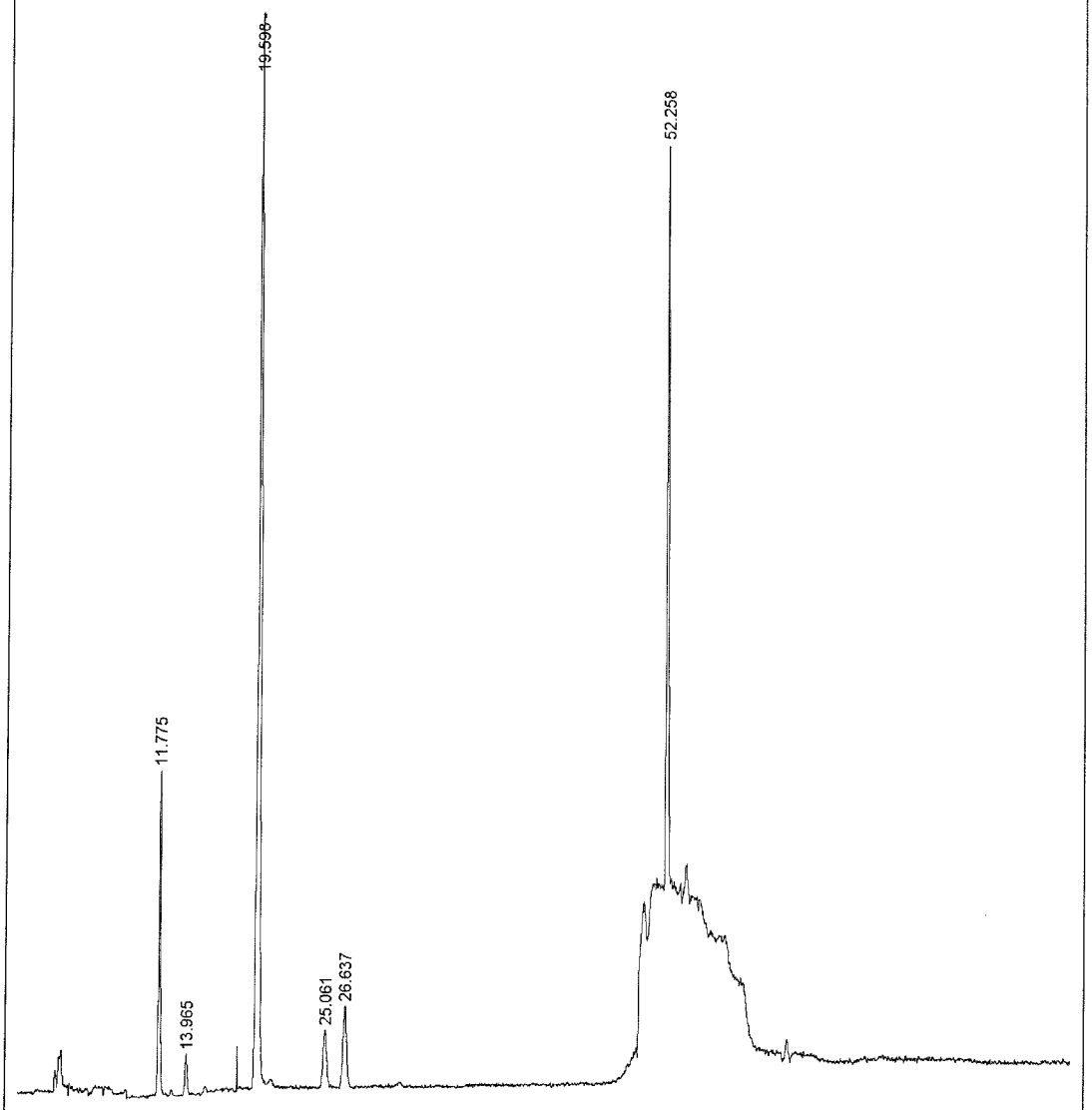
Mobile phase gradient program

Time (min)	A (%): Formic acid 0.1%	B (%): Acetonitrile
0	75	25
30	75	25
33	68	32
75	68	32

The assay shows that the compound observed on LC-MS system at 11.77min is the same as the compound at 3.5min, in EXAMPLE 23 ($C_{56}H_{90}O_{33}$; later confirmed as reb *M2*), and the compound observed at 26.64 min is the same as the compound at 7.6min, in EXAMPLE 23 ($C_{50}H_{80}O_{28}$; reb *UNK;* later confirmed as reb *D2*). Other isomers of reb M were observed at 13.96min and also another isomer form of reb *D* was observed at

25.06min. All observed compounds occurred in the extract of *Stevia rebaudiana* Bertoni plant.

SAMPLE : UGTSL2 T45h
Gene references: UGTSL2 (XP_004250485.1)



MSD SIM Results		
Compound	**Retention time**	**MW**
Unknown@RT11.77	11.775	1,291
Unknown@RT13.96	13.965	1,291
Rebaudioside *D*	19.598	1,129
Unknown@RT25.06	25.061	1,129
Unknown@RT26.64	26.637	1,129
Rebausioside *A*	52.258	967

EXAMPLE 32

In vivo preparation and activity determination of UGTLB

SEQ ID 10

UGTLB (GI_209954733 / BAG80557.1) amino acid sequence
MGTEVTVHKNTLRVLMFPWLAYGHISPFLNVAKKLVDRGFLIYLCSTAINLKSTIK
KIPEKYSDSIQLIELHLPELPELPPHYHTTNGLPPHLNHTLQKALKMSKPNFSKILQ
NLKPDLVIYDLLQQWAEGVANEQNIPAVKLLTSGAAVLSYFFNLVKKPGVEFPFP
AIYLRKNELEKMSELLAQSAKDKEPDGVDPFADGNMQVMLMSTSRIIEAKYIDYF
SGLSNWKVVPVGPPVQDPIADDADEMELIDWLGKKDENSTVFVSFGSEYFLSKED
REEIAFGLELSNVNFIWVARFPKGEEQNLEDALPKGFLERIGDRGRVLDKFAPQPRI
LNHPSTGGFISHCGWNSVMESVDFGVPIIAMPIHLDQPMNARLIVELGVAVEIVRD
DYGKIHREEIAEILKDVIAGKSGENLKAKMRDISKNLKSIRDEEMDTAAEELIQLC
KNSPKLK

The pET30A+ vector containing the UGTLB gene was introduced in *E. coli* Bl21(DE3) by heat shock. The obtained cells were grown in petri-dishes in the presence of Kanamycin and suitable colonies were selected and allowed to grow in liquid LB medium (erlenmeyer flasks). Glycerol was added to the suspension as cryoprotecteur and 400 µL aliquots were stored at -20°C and at -80°C.

The storage aliquots of *E. coli* BL21(DE3) containing the pET30A+_UGTLB plasmids were thawed and added to 30 mL of LBGKP medium (20 g/L Luria Broth Lennox; 50 mM PIPES buffer pH 7.00; 50 mM Phosphate buffer pH 7.00; 2.5 g/L glucose and 50 mg/L of Kanamycine). This culture was allowed to shake at 135 rpm at 30°C for 8 h.

The production medium contained 60 g/L of overnight express instant TB medium (Novagen), 10 g/L of glycerol and 50 mg/L of Kanamycine. The preculture was added to 200 mL of this medium and the solution was allowed to stir at 20°C while taking samples to measure the OD and pH. The culture gave significant growth and a good OD was obtained. After 40 h, the cells were harvested by centrifugation and frozen to obtain 5.7 g of cell wet weight.

Lysis was performed on 1.2 g of cells by addition of 6 mL Bugbuster Master mix (Novagen) and the lysate was recovered by centrifugation and used fresh.

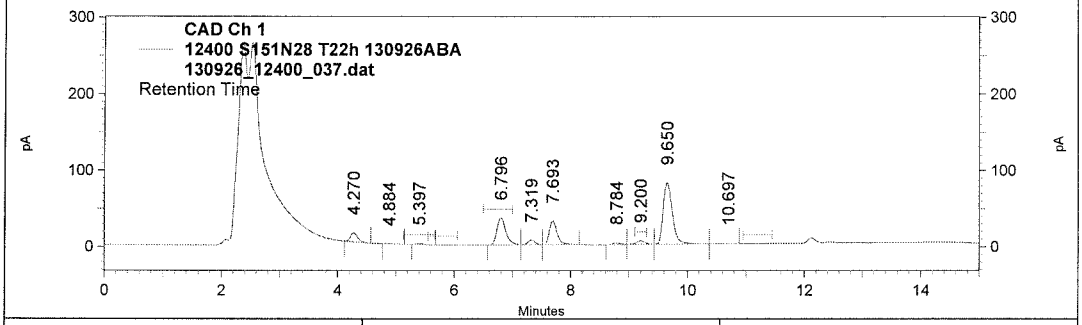
Determination of activity for Stevioside to Rebaudioside E conversion with UGTLB

Activity tests were performed at 3 mL scale with 600 µL of lysate for the transformation of Stevioside using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC as shown in Figures 64-66. The corresponding chromatograms are depicted below.

Enzyme internal reference	GI Number	Version	Stevioside conv.[1] (reaction time)	Rebaudioside E formation[1]
UGTLB	209954733	BAG80557.1	89% (22 h.)	3%

Note: [1]Based on initial concentration of Stevioside

SAMPLE : 12400 S151N28 T22h 130926ABA
Gene references: UGTLB (BAG80557.1)
Filename : 130926_12400_037.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Unknown@4.27	4.270	101,580,340
Unknown@4.88	4.884	2,979,482
Rebaudioside E	5.397	13,747,837
Unknown@6.80	6.796	378,936,196
Unknown@7.32	7.319	54,838,779
Unknown@7.69	7.693	291,189,747
Unknown@8.78	8.784	21,079,018
Stevioside	9.200	50,143,248
Unknown@9.65	9.650	888,211,556
Unknown@10.70	10.697	5,878,160
Totals		1,808,584,363

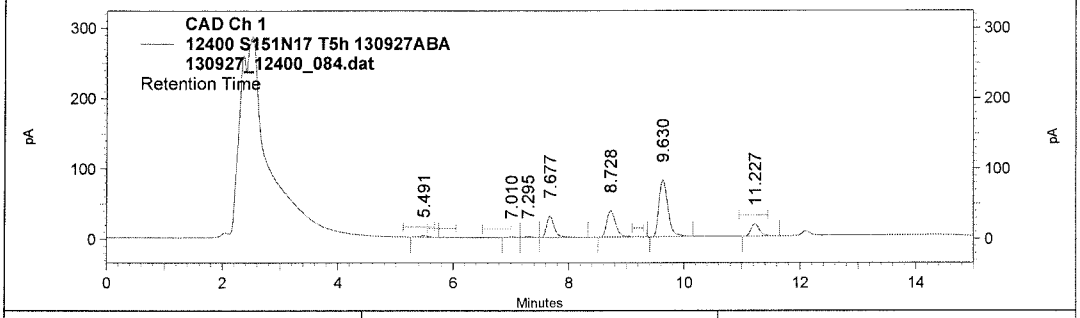
Determination of activity for Rubusoside to Rebaudioside E conversion with UGTLB

Activity tests were performed at 3 mL scale with 600 µL of lysate for the transformation of Rubusoside using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC. The corresponding chromatograms are depicted below.

Enzyme internal reference	GI Number	Version	Rubusoside conv.[1] (reaction time)	Rebaudioside E formation[1]
UGTLB	209954733	BAG80557.1	65 % (5 h.)	4 %

Note: [1]Based on initial concentration of Rubusoside

SAMPLE : 12400 S151N17 T5h 130927ABA
Gene references: UGTLB (BAG80557.1)
Filename : 130927_12400_084.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside E	5.491	21921232
Unknown@7.01	7.010	9764063
Unknown@7.29	7.295	12510947
Unknown@7.68	7.677	283386906
Unknown@8.73	8.728	402240506
Unknown@9.63	9.630	878990745
Rubusoside	11.227	176000085
Totals		1784814484

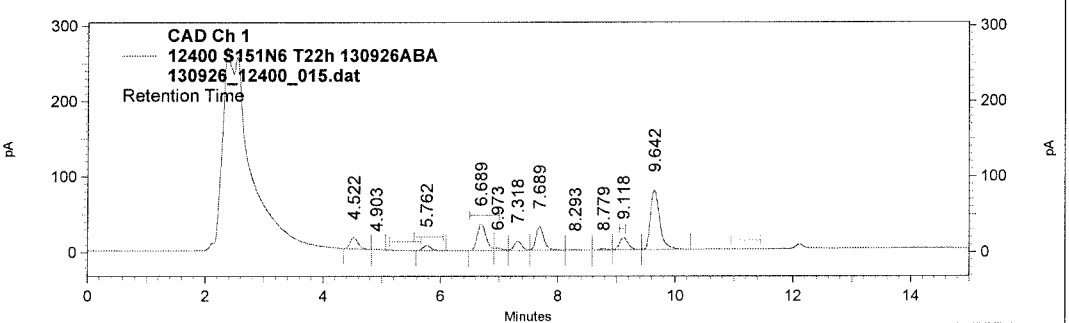
Determination of activity for Rebaudioside A to Rebaudioside D conversion with UGTLB

Activity tests were performed at 3 mL scale with 600 µL of lysate for the transformation of Rebaudioside A using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC. The corresponding chromatogram after 23 h. of reaction is depicted below.

Enzyme internal reference	GI Number	Version	Rebaudioside A conv.[1] (reaction time)	Rebaudioside D formation[1]
UGTLB	209954733	BAG80557.1	72% (22 h.)	10%

Note: [1]Based on initial concentration of Rebaudioside A

SAMPLE : 12400 S154N6 T22h 130926ABA
Gene references: UGTLB (BAG80557.1)
Filename : 130926_12400_015.dat



CAD Ch 1 Results		

Compound	Retention time	Integration (area)
Unknown@4.42	4.522	137,916,950
Unknown@4.90	4.903	2,015,271
Rebaudioside D	5.762	59,876,764
Unknown@6.69	6.689	364,185,331
Unknown@6.97	6.973	26,368,965
Unknown@7.32	7.318	110,284,197
Unknown@7.69	7.689	294,579,799
Unknown@8.29	8.293	7,867,452
Unknown@8.78	8.779	15,928,550
Rebausioside A	9.118	165,602,247
Unknown@9.64	9.642	868,327,712
Totals		2,052,953,238

EXAMPLE 33

Determination of reaction products for Rubusoside and Stevioside conversion with UGTSL, UGTSL2, and UGTLB

Conversion of stevioside with UGTSL and UGTSL2 was conducted in similar manner to Example 28, and the conversion of rubusoside with UGTSL and UGTSL2 was conducted similarly to Example 29. Conversions of rubusoside and stevioside with UGTLB was conducted similarly to Example 32.

The reaction mixtures were analyzed by LCMS to determine all reaction products.

Rubusoside conversion products

Sample ID	UGT (reaction time)	LC-MS, peak area ratio (%)						
		Rub	Stev	Reb E	Reb D	Unknown peak #1 (MW804) RT 30.70min	Unknown peak #2 (MW804) RT 49.50min	Unknown peak #3 (MW804) RT 50.40min
S151N15	UGTSL2 (2hrs)	3.54	2.12	52.88	6.73	12.02	9.94	12.77
S151N17	UGTLB (5hrs)	13.49	ND	9.21	1.29	4.07	66.67	5.27
S151N22	UGTSL (45hrs)	7.82	2.37	35.88	3.45	20.38	27.75	2.35

Stevioside conversion products

Sample ID	UGT (reaction time)	LC-MS, peak area ratio (%)					
		Stev	Reb E	Reb D	Unknown peak #1 (MW966) RT=22.60 min	Unknown peak #2 (MW966) RT=26.50 min	Unknown peak #3 (MW966) RT=29.50 min
S151N26	UGTSL2 (2hrs)	20.01	42.56	1.70	4.48	5.56	25.70
S151N28	UGTLB (2hrs)	43.11	3.12	ND	ND	53.78	ND
S151N33	UGTSL (22hrs)	25.24	49.68	0.54	3.97	20.56	ND

It can be seen that amongst Rubusoside conversion products, besides Stevioside, Reb E and Reb D, there are at least 3 additional compounds with Molecular Weight of 804. The retention time of these compounds do not match with Reb B which is known to have same Molecular Weight as Stevioside. Since these compounds have same molecular weight with Stevioside it can be assumed that these novel steviol glycosides are isomers of Stevioside.

On the other hand amongst Stevioside conversion products, besides Reb E and Reb D, there are at least 3 additional compounds with Molecular Weight of 966. The retention time of these compounds do not match with Reb A which is known to have same Molecular Weight as Reb E. Since these compounds have same molecular weight with Reb A and Reb E it can be assumed that these novel steviol glycosides are isomers of Reb A (Reb E).

EXAMPLE 34

In vivo production of UGT76G1 in *S. cerevisiae*

SEQ ID 11

UGT76G1 [*Stevia rebaudiana*] (gi_37993653 /gb_AAR06912.1)

MENKTETTVRRRRRIILFPVPFQGHINPILQLANVLYSKGFSITIFHTNFNKPKTSNY
PHFTFRFILDNDPQDERISNLPTHGPLAGMRIPIINEHGADELRRELELLMLASEEDE
EVSCLITDALWYFAQSVADSLNLRRLVLMTSSLFNFHAHVSLPQFDELGYLDPDD
KTRLEEQASGFPMLKVKDIKSAYSNWQILKEILGKMIKQTKASSGVIWNSFKELEE
SELETVIREIPAPSFLIPLPKHLTASSSSLLDHDRTVFQWLDQQPPSSVLYVSFGSTS
EVDEKDFLEIARGLVDSKQSFLWVVRPGFVKGSTWVEPLPDGFLGERGRIVKWVP
QQEVLAHGAIGAFWTHSGWNSTLESVCEGVPMIFSDFGLDQPLNARYMSDVLKV
GVYLENGWERGEIANAIRRVMVDEEGEYIRQNARVLKQKADVSLMKGGSSYESL
ESLVSYISSL

The above mentioned amino acid sequence was codon optimized for expression in *S. cerevisiae*. Furthermore the yeast consensus sequence AACACA was added before the ATG start codon. The synthetic gene was subcloned in the pYES2 vector using Hind III and Xba I restriction sites. The pYES2_UGT76G1_Sc vector was used to transform chemically competent *S. cerevisiae* INVSc1 cells (Invitrogen).

The cells were grown on a solid synthetic minimal medium containing 2% glucose lacking Uracil and a single colony was picked and allowed to grow in liquid synthetic minimal medium lacking Uracil (SC-U containing 2% glucose). After centrifugation, the cells were suspended with SC-U (containing 2% glucose) and 60% glycerol/water. Aliquots were stored at -80°C and one aliquot was used to start a culture in SC-U (containing 2%

glucose) for 43 h at 30°C. Part of this culture was centrifuged and suspended in induction medium (SC-U containing 2% galactose) for 19h30 at 30 °C.

Cells were obtained by centrifugation and lysis with five volumes of CelLytic ™ Y Cell Lysis Reagent (Sigma). The lysates were used directly for activity testing (UGT76G1_Sc).

EXAMPLE 35

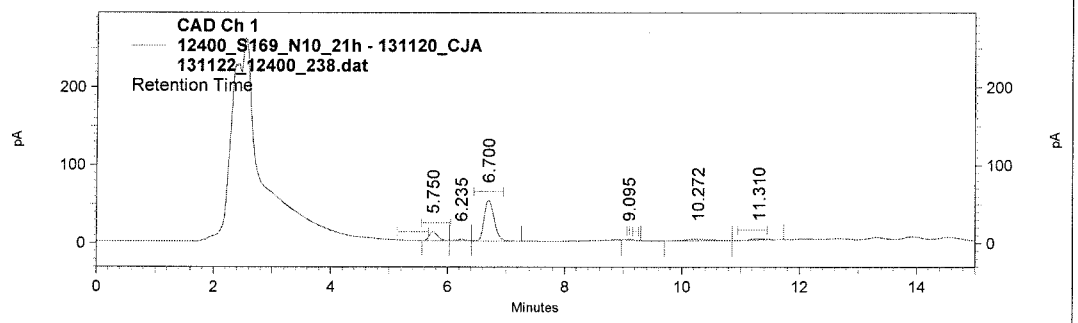
Determination of activity of UGT76G1_Sc for the conversion of Rebaudioside D to Rebaudioside M

UGT76G1_Sc was prepared according to EXAMPLE 34. Activity tests were performed at 2 mL scale with 200 µL of lysate for the transformation of Rebaudioside D using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer at pH 7.2. Samples were taken and analyzed by HPLC as shown in Figure 67. The corresponding chromatogram is depicted below.

Enzyme internal reference	Rebaudioside D conv.[1] (reaction time)	Rebaudioside M selectivity[1]
UGT76G1_Sc	85% (21h.)	100%

Note: [1]Based on initial concentration of Rebaudioside D

SAMPLE : 12400 S169N10 T21h 131119CJA
Gene references: UGT76G1_Sc
Filename : 131122_12400_238.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Rebaudioside D	5.750	112,094,430
Unknown@6.23	6.235	17,886,043
Rebaudioside M	6.700	616,583,935
Rebaudioside A	9.095	11,183,884
Unknown@10.27	10.272	62,863,156
Unknown@11.31	11.310	35,839,478
Total		856,450,926

EXAMPLE 36

In vivo production of UGTSL in *S. cerevisiae*

SEQ ID 12

UGTSL [*Solanum lycopersicum*] (gi_460409128 / XP_004249992.1

MSPKLHKELFFHSLYKKTRSNHTMATLKVLMFPFLAYGHISPYLNVAKKLADRGF
LIYFCSTPINLKSTIEKIPEKYADSIHLIELHLPELPQLPPHYHTTNGLPPNLNQVLQK
ALKMSKPNFSKILQNLKPDLVIYDILQRWAKHVANEQNIPAVKLLTSGAAVFSYFF
NVLKKPGVEFPFPGIYLRKIEQVRLSEMMSKSDKEKELEDDDDDDDLLVDGNMQI
MLMSTSRTIEAKYIDFCTALTNWKVVPVGPPVQDLITNDVDDMELIDWLGTKDE
NSTVFVSFGSEYFLSKEDMEEVAFALELSNVNFIWVARFPKGEERNLEDALPKGFL
ERIGERGRVLDKFAPQPRILNHPSTGGFISHCGWNSAMESIDFGVPIIAMPMHLDQP
MNARLIVELGVAVEIVRDDDGKIHRGEIAETLKGVITGKTGEKLRAKVRDISKNLK
TIRDEEMDAAAEELIQLCRNGN

The above mentioned amino acid sequence was codon optimized for expression in *S. cerevisiae*. Furthermore the yeast consensus sequence AACACA was added before the ATG start codon. The synthetic gene was subcloned in the pYES2 vector using Hind III and Xba I restriction sites. The pYES2_UGTSL_Sc vector was used to transform chemically competent *S. cerevisiae* INVSc1 cells (Invitrogen).

The cells were grown on a solid synthetic minimal medium containing 2% glucose, lacking Uracil and a single colony was picked and allowed to grow in liquid synthetic minimal medium lacking Uracil (SC-U containing 2% glucose). After centrifugation, the cells were suspended with SC-U (containing 2% glucose) and 60% glycerol/water. Aliquots were stored at -80°C and one aliquot was used to start a culture in SC-U (containing 2% glucose) for 43 h at 30°C. Part of this culture was centrifuged and suspended in induction medium (SC-U containing 2% galactose) for 19h30 at 30°C.

Cells were obtained by centrifugation and lysis with five volumes of CelLytic ™ Y Cell Lysis Reagent (Sigma). The lysates were used directly for activity testing (UGTSL_Sc).

EXAMPLE 37

Determination of activity of UGTSL_Sc for the conversion of Rebaudioside A to Rebaudioside D

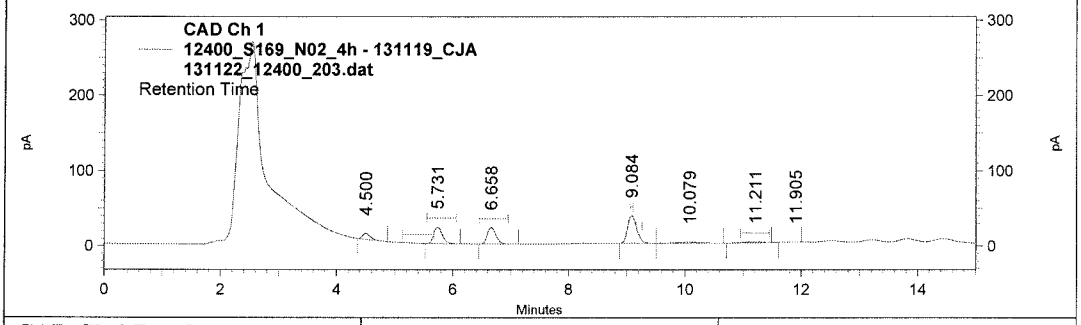
UGTSL_Sc was prepared according to EXAMPLE 36. Activity tests were performed at 2 mL scale with 200 µL of lysate for the transformation of Rebaudioside A using 0.5 mM of substrate, 2.5 mM of UDP-Glucose and 3 mM $MgCl_2$ in 50 mM Sodium Phosphate buffer

at pH 7.2. Samples were taken and analyzed by HPLC as shown in Figure 68. The corresponding chromatogram is depicted below.

Enzyme internal reference	Rebaudioside A conv.[1] (reaction time)	Rebaudioside D selectivity[1]
UGTSL_Sc	46% (4h)	42 %

Note: [1]Based on initial concentration of Rebaudioside A

SAMPLE : 12400 S169N02 T4h 131119CJA
Gene references: UGTSL_Sc
Filename : 131122_12400_203.dat



CAD Ch 1 Results		
Compound	**Retention time**	**Integration (area)**
Unknown@4.50	4.500	75,046,986
Rebaudioside D	5.731	223,409,643
Unknown@6.66	6.658	228,651,278
Rebaudioside A	9.084	404,642,305
Unknown@10.08	10.079	43,992,253
Unknown@11.21	11.211	29,776,761
Unknown@11.90	11.905	2,185,316
Total		1,007,704,542

EXAMPLE 38

Isolation of Rebaudioside *M*

The amount of the product mixture of Example 14 was not large enough to separate via preparative HPLC methods. Accordingly, analytical HPLC with a series of injections was used to separate the components of the mixture. Separation was conducted according to the method described above in Example 14 to provide two fractions corresponding to the two main peaks in the HPLC trace of FIG. 5: Fraction A (retention time 24.165 minutes) and Fraction B (retention time 31.325 minutes).

The retention time of Fraction A was consistent with reb *D*, indicating unreacted starting material from the biotransformation reaction.

The retention time of purified Fraction B (FIG. 6) was consistent with reb *M*, indicating successful biotransformation from reb *D*. The identity of the material collected in Fraction B as reb *M* was confirmed by co-injection of purified Fraction B with a reb *M* standard (available from PureCircle, HPLC trace of reb *M* standard shown in FIG. 7). Both

Fraction B and the reb *M* standard were found to elute at the same retention time (FIG. 8), indicating Fraction B was reb *M*.

The identity of Fraction B as reb *M* was also separately confirmed by NMR and HRMS. For sampling, Fraction B was concentrated under rotary evaporator, freeze dried and dried for 40 h at 40 °C.

The NMR sample was dissolved in deuterated pyridine (C_5D_5N) and spectra were acquired on a Varian Unity Plus 600 MHz instrument using standard pulse sequences. The NMR spectra of Fraction B was compared to the NMR spectra of reb *M*. An overlay of the two spectra (FIG. 9) showed consistency of peaks of Fraction B with reb *M*. A table of the NMR assignments for reb *M* is shown below:

^{1}H and ^{13}C NMR spectral data for Rebaudioside *M* in C_5D_5N [a-c].

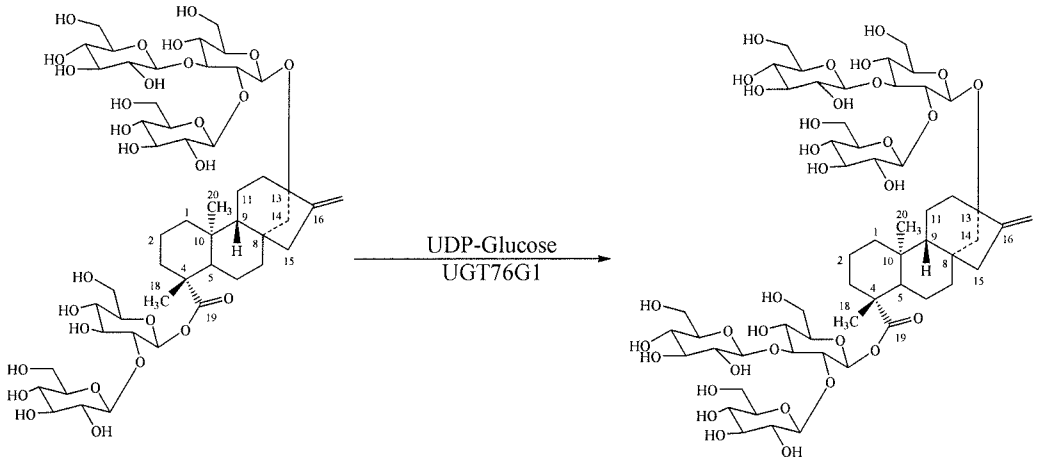
Position	^{13}C NMR	^{1}H NMR
1	40.3	0.75 t (13.2) 1.76 m
2	19.6	1.35 m 2.24 m
3	38.4	1.01 m 2.30 d (13.3)
4	44.3	---
5	57.4	1.06 d (12.8)
6	23.5	2.23 m 2.41 q (13.2)
7	42.6	1.41 m 1.80 m
8	41.2	---
9	54.3	0.91 d (7.7)
10	39.7	---
11	20.2	1.65 m 1.75 m
12	38.5	1.86 m 2.73 m
13	87.6	---
14	43.3	2.02 m 2.74 m
15	46.5	1.88 d (16.4) 2.03 m
16	153.3	---
17	104.9	4.90 s 5.69 s
18	28.2	1.32 s
19	176.9	---
20	16.8	1.38 s

1′	94.9	6.39 d (8.2)
2′	76.9	4.51 t (8.5)
3′	88.6	5.09 t (8.5)
4′	70.1	4.18 m
5′	78.4	4.13 m
6′	61.8	4.20 m 4.31 m
1″	96.2	5.46 d (7.1)
2″	81.4	4.13 m
3″	87.9	4.98 t (8.5)
4″	70.4	4.07 t (9.6)
5″	77.7	3.94 m
6″	62.6	4.19 m 4.32 m
1‴	104.8	5.48 d (7.7)
2‴	75.8	4.15 m
3‴	78.6	4.13 m
4‴	73.2	3.98 m
5‴	77.6	3.74 ddd (2.8, 6.4, 9.9)
6‴	64.0	4.27 m 4.51m
1⁗	103.9	5.45 d (7.5)
2⁗	75.6	3.98 m
3⁗	77.8	4.50 t (7.8)
4⁗	71.3	4.14 m
5⁗	78.0	3.99 m
6⁗	62.1	4.20 m 4.32 m
1′′′′′	104.2	5.81 d (7.2)
2′′′′′	75.5	4.20 m
3′′′′′	78.4	4.20 m
4′′′′′	73.6	4.10 m

5'''''	77.8	3.90 ddd (2.8, 6.4, 9.9)
6'''''	64.0	4.32 m 4.64 d (10.3)
1'''''''	104.1	5.31 d (8.0)
2'''''''	75.5	3.95 m
3'''''''	78.0	4.37 t (9.1)
4'''''''	71.1	4.10 m
5'''''''	78.1	3.85 ddd (1.7, 6.1, 9.9)
6'''''''	62.1	4.10 m 4.32 m

[a] assignments made on the basis of COSY, HMQC and HMBC correlations; [b] Chemical shift values are in δ (ppm); [c] Coupling constants are in Hz.

HRMS (FIG. 10) was generated with a Waters Premier Quadropole Time-of-Flight (Q-TOF) mass spectrometer equipped with an electrospray ionization source operated in the positive-ion mode. The sample was dissolved in methanol and eluted in 2:2:1 methanol: acetonitrile: water and introduced via infusion using the onboard syringe pump. The presence of reb *M* was confirmed by a $[M+Na]^+$ adduct at m/z 1313.5265, which corresponds to a molecular formula of $C_{56}H_{90}O_{33}$



Rebaudioside D
Chemical Formula: $C_{50}H_{80}O_{28}$
Molecular Weight: 1128

Rebaudioside M
Chemical Formula: $C_{56}H_{90}O_{33}$
Molecular Weight: 1290

EXAMPLE 39

Isolation and Characterization of Reb *D2*

Crude Reaction Sample. The sample, Lot CB-2977-106, used for isolation, was prepared according to Example 22 with UGTSL (GI #460409128).

HPLC Analysis. Preliminary HPLC analyses of samples were performed using a Waters 2695 Alliance System with the following method: Phenomenex Synergi Hydro-RP, 4.6 × 250 mm, 4 µm (p/n 00G-4375-E0); Column Temp: 55 °C; Mobile Phase A: 0.0284% ammonium acetate (NH_4OAc) and 0.0116% acetic acid (HOAc) in water; Mobile Phase B: Acetonitrile (MeCN); Flow Rate: 1.0 mL/min; Injection volume: 10 µL. Detection was by UV (210 nm) and CAD.

Gradient:

Time (min)	%A	%B
0.0 – 8.5	75	25
10.0	71	29
16.5	70	30
18.5 – 24.5	66	34
26.5 – 29.0	48	52
31 – 37	30	70
38	75	25

Analyses of semi-preparative purification fractions were performed with the following method: Waters Atlantis dC18, 4.6 × 100 mm, 5 µm (p/n 186001340); Mobile Phase A: 25% MeCN in water; Mobile Phase B: 30% MeCN in water; Flow Rate: 1.0 mL/min; Injection volume: 10 µL. Detection was by CAD.

Gradient:

Time (min)	%A	%B
0.0 – 5.0	100	0
20	20	80
25	20	80
30	100	0

LC-MS. Preliminary analysis of the semi-synthetic steviol glycoside mixture was carried out on a Waters AutoPurification HPLC/MS System with a Waters 3100 Mass Detector operating in negative ion mode. Analysis of the sample was performed using the following method: Phenomenex Synergi Hydro-RP, 4.6 × 250 mm, 4 µm (p/n 00G-4375-E0); Column Temp: 55 °C; Mobile Phase A: 0.0284% NH_4OAc and 0.0116% HOAc in water; Mobile Phase B: Acetonitrile; Flow Rate: 1.0 mL/min; Injection volume: 10 µL. Detection was by UV (210 nm), and MSD (-ESI m/z 500 – 2000). Gradient conditions were as listed above.

Isolation by HPLC. The purification was performed in two steps. The first method used for the semi-preparative purification is summarized below. Column: Waters Atlantis dC18, 30 × 100 mm, 5 µm (p/n 186001375); Mobile Phase A: 25% MeCN in water;

Mobile Phase B: 30% MeCN in water; Flow Rate: 45 mL/min; Injection load: 160 mg dissolved in 20 mL of water. Detection was by UV (205 nm).
Gradient:

Time (min)	%A	%B
0.0 – 5.0	100	0
20	20	80
25	20	80
30	100	0

The secondary purification used the same column and conditions, but isocratic mobile phase: 20% MeCN in water.

Purification from Natural Extracts. The purification was performed in three steps. The first method used for the preparative purification is summarized below. Primary Process: Waters Symmetry C18, 50 × 250 mm, 7 µm (p/n WAT248000); Isocratic mobile phase: 50% methanol (MeOH) in water with 0.05% HOAc; Flow Rate: 85 mL/min; Injection load: 6 g crude extract dissolved in 50 mL of mobile phase. Detection was by UV (210 nm). Following the elution of target analytes, the column was flushed with 85% MeOH in water.

Secondary Process: Waters Symmetry Shield RP18, 50 × 250 mm, 7 µm (p/n WAT248000); Isocratic mobile phase: 20% MeCN in water; Flow Rate: 100 mL/min; Injection load: 0.5 g primary fraction dissolved in 30 mL of water. Detection was by UV (210 nm).

Tertiary Process: Waters Symmetry Shield RP18, 50 × 250 mm, 7 µm (p/n WAT248000); Isocratic mobile phase: 20% MeCN in water; Flow Rate: 100 mL/min; Injection load: 0.5 g secondary fraction dissolved in 30 mL of water. Detection was by UV (210 nm).

MS and MS/MS. MS and MS/MS data were generated with a Waters QT of Premier mass spectrometer equipped with an electrospray ionization source. Samples were analyzed by negative ESI. Samples were diluted with H_2O:acetonitrile (1:1) by 50 fold and introduced

via infusion using the onboard syringe pump. The samples were diluted to yield good s/n which occurred at an approximate concentration of 0.01 mg/mL.

NMR. The sample was prepared by dissolving 1 – 2 mg in 150 µL of pyridine-d_5 and NMR data were acquired on a Bruker Avance 500 MHz instrument with a 2.5 mm inverse detection probe. The ^{1}H NMR spectrum was referenced to the residual solvent signal (δ_H 8.74 and δ_C 150.35 for pyridine-d_5).

Results and Discussion

Isolation and Purification. Isolation was performed on steviol glycoside mixture, Lot number CB-2977-106, prepared according to Example 22 with UGTSL (GI #460409128) The material was analyzed by LC-MS using the method described above and results are provided in Figure 11. The targeted peak of interest was that at 7.7 min in the TIC chromatogram. The mass spectrum of this peak provided a $[M-H]^-$ ion at *m/z* 1127.6. The provided sample was preliminarily processed in a single injection (160 mg) using the first method condition provided above. This method fractionated the material into 'polar' and 'non-polar' mixtures of glycosides. The 'polar' mixture was then reprocessed using the second-step conditions above. The semi-preparative HPLC trace is provided in Figure 12. From this semi-preparative collection, the compound was isolated with a purity >99% (CAD, AUC). The fraction analysis is provided in Figure 13. Following the purification, the combined fractions were concentrated by rotary evaporation at 35 °C and lyophilized. Approximately 1 – 2 mg was obtained for characterization.

Mass Spectrometry. The ESI- TOF mass spectrum acquired by infusing a sample showed a $[M-H]^-$ ion at *m/z* 1127.4709. The mass of the $[M-H]^-$ ion was in good agreement with the molecular formula $C_{50}H_{80}O_{28}$ (calcd for $C_{50}H_{79}O_{28}$: 1127.4758, error: -4.3 ppm). The MS data confirmed a nominal mass of 1128 Daltons with the molecular formula, $C_{50}H_{80}O_{28}$.

The MS/MS spectrum (selecting the $[M-H]^-$ ion at *m/z* 1127.5 for fragmentation) indicated the loss of two glucose units and sequential loss of three glucose moieties at *m/z* 641.3187, 479.2655 and 317.2065.

NMR Spectroscopy. A series of NMR experiments including ^{1}H NMR (Figure 14), ^{13}C NMR (Figure 15 and 16), ^{1}H-^{1}H COSY (Figure 17), HSQC-DEPT (Figure 18), HMBC (Figures 19 and 20), NOESY (Figure 21) and 1D-TOCSY (Figure 22-26) were performed to allow assignment of the compound. In the ^{1}H NMR acquired after ~46 hrs of sample preparation (Figures 27-28), the anomeric resonance at δ_H 5.04 is resolved which was obscured by the solvent (HOD) in the original spectrum (Figure 14)

The ^{1}H, ^{1}H-^{1}H COSY, ^{1}H-^{13}C HSQC-DEPT and ^{1}H-^{13}C HMBC NMR data indicated that the central core of the glycoside is a diterpene. The presence of five anomeric protons observed in the ^{1}H and ^{1}H-^{13}C HSQC-DEPT spectra confirm five sugar units in the structure. The methylene ^{13}C resonance at δ_C 69.9 in the ^{1}H-^{13}C HSQC-DEPT spectrum indicated the presence of a 1→6 sugar linkage in the structure. The linkages of sugar units were assigned using ^{1}H-^{13}C HMBC and 1D-TOCSY correlations.

A HMBC correlation from the methyl protons at δ_H 1.29 to the carbonyl at δ_C 177.7 allowed assignment of one of the tertiary methyl groups (C-18) as well as C-19 and provided a starting point for the assignment of the rest of the aglycone. Additional HMBC correlations from the methyl protons (H-18) to carbons at δ_C 38.9, 45.0, and 57.8 allowed assignment of C-3, C-4, and C-5. Analysis of the ^{1}H-^{13}C HSQC-DEPT data indicated that the carbon at δ_C 38.9 was a methylene group and the carbon at δ_C 57.8 was a methine which were assigned as C-3 and C-5, respectively. This left the carbon at δ_C 45.0, which did not show a correlation in the HSQC-DEPT spectrum, to be assigned as the quaternary carbon, C-4. The ^{1}H chemical shifts for C-3 (δ_H 0.98 and 2j.36) and C-5 (δ_H 1.04) were assigned using the HSQC-DEPT data. A COSY correlation between one of the H-3 protons (δ_H 0.98) and a proton at δ_H 1.43 allowed assignment of one of the H-2 protons which in turn showed a correlation with a proton at δ_H 0.75 which was assigned to C-1. The remaining ^{1}H and ^{13}C chemical shifts for C-1 and C-2 were then assigned on the basis of additional COSY and HSQC-DEPT correlations and are summarized in the table below.

^{1}H and ^{13}C NMR (500 and 125 MHz, pyridine-d_5), Assignments of Reb *D2*.

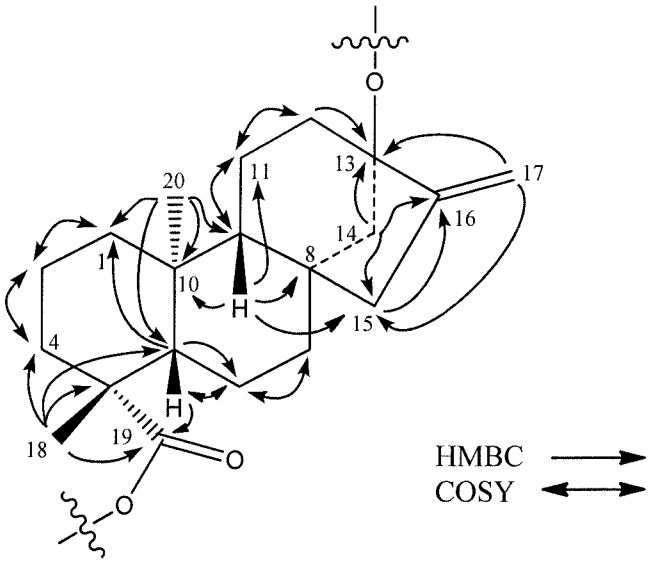
	Reb *D2*	
Position	^{13}C	^{1}H
1	41.3	0.75 t (11.0)

		1.76 m
2	19.9	1.43 m 2.20 m
3	38.9	0.98 m 2.36 d (12.1)
4	45.0	---
5	57.8	1.04 d (12.5)
6	22.7	1.92 m 2.43 m
7	42.2	1.22 m 1.30 m
8	43.1	---
9	54.5	0.88 brs
10	40.3	---
11	21.1	1.65 m 1.69 m
12	37.5	1.99 m 2.25 m
13	87.1	---
14	44.5	1.80 d (11.7) 2.65 d (11.7)
15	48.3	1.31 m 2.04 brs
16	154.7	---
17	105.2	5.01 s 5.64 s
18	28.8	1.29 s
19	177.7	---
20	16.0	1.30 s

The other tertiary methyl singlet, observed at δ_H 1.30 showed HMBC correlations to C-1 and C-5 and was assigned as C-20. The methyl protons showed additional HMBC correlations to a quaternary carbon (δ_C 40.3) and a methine carbon (δ_C 54.5) which were assigned as C-10 and C-9, respectively. COSY correlations between H-5 (δ_H 1.04) and protons at δ_H 1.92 and 2.43 then allowed assignment of the H-6 protons which in turn showed correlations to protons at δ_H 1.22 and 1.30 which were assigned to C-7. The ^{13}C chemical shifts for C-6 (δ_C 22.7) and C-7 (δ_C 42.2) were then determined from the HSQC-DEPT data. COSY correlations between H-9 (δ_H 0.88) and protons at δ_H 1.65 and 1.69 allowed assignment of the H-11 protons which in turn showed COSY correlations to protons at δ_H 1.99 and 2.25 which were assigned as the H-12 protons. The HSQC-DEPT data was then used to assign C-11 (δ_C 21.1) and C-12 (δ_C 37.5). HMBC correlations from the H-12 proton (δ_H 2.25) to carbons at δ_C 87.1 and 154.7 allowed assignment of C-13 and C-16, respectively. The olefinic protons observed at δ_H 5.01 and 5.64 showed HMBC correlations to C-13 and were assigned to C-17 (δ_C 105.2 via HSQC-DEPT). The olefinic protons H-17 and the methine proton H-9 showed HMBC correlations to a carbon at δ_C 48.3 which was assigned as C-15. An additional HMBC correlation from H-9 to a methylene carbon at δ_C 44.5 then allowed assignment of C-14. The ^1H chemical shifts at C-14 (δ_H 1.80 and 2.65) and C-15 (δ_H 1.31 and 2.04) were assigned using the HSQC-DEPT data.

Correlations observed in the NOESY spectrum were used to assign the relative stereochemistry of the central diterpene core. In the NOESY spectrum, NOE correlations were observed between H-14 and H-20 indicating that H-14 and H-20 are on the same face of the rings. Similarly, NOE correlations were observed between H-9 and H-5; H-9 and H-18 as well as H-5 and H-18 but NOE correlations were not observed between H-9 and H-14 indicating that H-5, H-9 and H-18 were on the opposite face of the rings compared to H-14 and H-20 as presented in Figure 21. These data thus indicated that the relative stereochemistry in the central core was retained during the glycosylation step.

The key HMBC and COSY correlations used to assign the aglycone region are provided below:



Analysis of the ^{1}H-^{13}C HSQC-DEPT data confirmed the presence of five anomeric protons. Three of the anomeric protons were well resolved at δ_H 6.02 (δ_C 96.1), 5.57 (δ_C 105.3), and 5.34 (δ_C 105.3) in the ^{1}H NMR spectrum. The remaining two anomeric protons observed at δ_H 5.04 (δ_C 105.6) and 5.07 (δ_C 98.7) which were obscured by solvent (HOD) resonance in the ^{1}H spectrum were identified by ^{1}H-^{13}C HSQC-DEPT data. The anomeric proton observed at δ_H 6.02 showed HMBC correlation to C-19 which indicated that it corresponds to the anomeric proton of Glc_I. Similarly, the anomeric proton observed at δ_H 5.07 showed an HMBC correlation to C-13 allowing it to be assigned as the anomeric proton of Glc_{II}.

The Glc_I anomeric proton (δ_H 6.02) showed a COSY correlation to a proton at δ_H 4.07 was assigned as Glc_I H-2 which in turn showed a COSY correlation to a proton at δ_H 4.22 (Glc_I H-3) which showed a COSY correlation with a proton at δ_H 4.12 (Glc_I H-4). Due to data overlap, the COSY spectrum did not allow assignment of H-5 or the H-6 protons. Therefore, a series of 1D-TOCSY experiments were performed using selective irradiation of the Glc_I anomeric proton with several different mixing times. In addition to confirming the assignments for Glc_I H-2 through H-4, the 1D-TOCSY data showed a proton at δ_H 4.04 assigned as Glc_I H-5 and a proton at δ_H 4.68 assigned as one of the Glc_I H-6 protons. The latter proton was also used for 1D-TOCSY experiments. The selective irradiation of H-6 with several different mixing times also confirmed the assignment of Glc_I H-1 to H-5 as well as the remaining methylene proton of H-6 (δ_H 4.30). Assignment

of the ^{13}C chemical shifts for Glc_I C-2 (δ_C 74.2), C-3 (δ_C 79.1), C-4 (δ_C 72.1), C-5 (δ_C 78.5), and C-6 (δ_C 69.9) was determined using the ^1H-^{13}C HSQC-DEPT data to complete the assignment of Glc_I. Furthermore, the presence of a methylene ^{13}C resonance at δ_C 69.9 in the ^1H-^{13}C HSQC-DEPT spectrum indicated a 1→6 sugar linkage of Glc_I in the structure.

Out of four remaining unassigned glucose moieties, one was assigned as a substituent at C-6 of Glc_I on the basis of ^1H-^{13}C HSQC-DEPT, HMBC, and 1D-TOCSY correlations. The relatively downfield shift of a methylene ^{13}C resonance of Glc_I at δ_C 69.9 in the HSQC-DEPT spectrum indicated a 1→6 sugar linkage of Glc_I. The anomeric proton observed at δ_H 5.04 showed HMBC correlation to Glc_I C-6 and was assigned as the anomeric proton of Glc_V. Similarly, methylene protons of Glc_I showed HMBC correlations to anomeric carbon of Glc_V confirming the presence of a 1→6 sugar linkage between Glc_I and Glc_V. The Glc_V anomeric proton showed a COSY correlation to a proton at δ_H 4.00 which was assigned as Glc_V H-2 which in turn showed a COSY correlation to a proton at δ_H 4.22 (Glc_V H-3). Due to data overlap, the COSY spectrum did not allow assignment of Glc_V H-4 based on the COSY correlation of Glc_V H-3. However, in the HMBC spectrum, Glc_V H-3 showed a correlation to Glc_V C-5 (δ_C 78.9). In HSQC-DEPT spectrum, Glc_V C-5 showed a correlation to δ_H 3.89 (Glc_V H-5). The Glc_V H-5 showed COSY correlations to δ_H 4.21, 4.37, and 4.48. In the HSQC-DEPT spectrum, δ_H 4.21 showed a correlation to δ_C 71.4 (Glc_V H-4), while δ_H 4.37 and 4.48 showed a correlation to δ_C 63.1 and were assigned to Glc_V H-6a and H-6b, respectively. Assignment of the ^{13}C chemical shifts for Glc_V C-2 (δ_C 75.7) and C-3 (δ_C 79.1) was determined using the ^1H-^{13}C HSQC-DEPT data to complete the assignment of Glc_V.

A summary of the ^1H and ^{13}C chemical shifts for the glycoside at C-19 are shown in the following table:

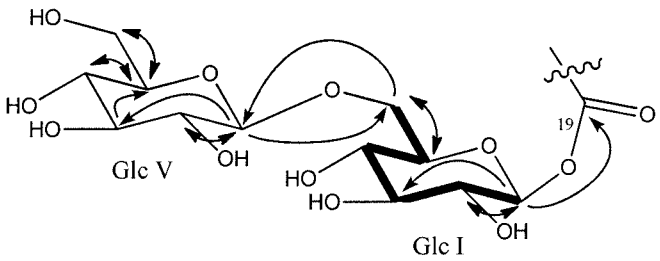
^1H and ^{13}C NMR (500 and 125 MHz, pyridine-d_5), Assignments of the reb *D2* C-19 glycoside.

	Reb *D2*	
Position	^{13}C	^1H

Glc_I-1	96.1	6.02 d (8.1)
Glc_I-2	74.2	4.07 m
Glc_I-3	79.1[#]	4.22 m[#]
Glc_I-4	72.1	4.12 m
Glc_I-5	78.5	4.04 m
Glc_I-6	69.9	4.30 m 4.68 d (10.7)
Glc_V-1	105.6	5.04 (8.1)
Glc_V-2	75.7	4.00 m
Glc_V-3	79.1[#]	4.22 m[#]
Glc_V-4	71.4	4.21 m
Glc_V-5	78.9	3.89 m
Glc_V-6	63.1	4.37 m 4.48 m

[#]^{1}H and ^{13}C values can be exchangeable between positions Glc_I-3, Glc_V-3 and Glc_{IV}-3.

A summary of the key HMBC, COSY, and 1D-TOCSY correlations used to assign the C-19 glycoside region are provided below.



Assignment of Glc_{II} was carried out in a similar manner. The Glc_{II} anomeric proton (δ_H 5.07) showed a COSY correlation to a proton at δ_H 4.37, assigned as Glc_{II} H-2,

which in turn showed a COSY correlation to a proton at δ_H 4.18 (Glc_{II} H-3). This latter proton showed an additional correlation with a proton at δ_H 3.88 (Glc_{II} H-4) which also showed a COSY correlation to a proton at δ_H 3.79 (Glc_{II} H-5). Glc_{II} H-5 also showed a COSY correlation to Glc_{II} H-6 protons (δ_H 4.08 and 4.46). Assignment of the ^{13}C chemical shifts for Glc_{II} C-2 (δ_C 81.3), C-3 (δ_C 88.4), C-4 (δ_C 71.1), C-5 (δ_C 77.9), and C-6 (δ_C 63.2) was determined using the HSQC-DEPT data. HMBC correlations from Glc_{II} H-3 to C-2 and C-4 and also from Glc_{II} H-4 to C-2 and C-5 confirmed the assignments made above. Additional HMBC correlations of Glc_{II} H-4 to Glc_{II} C-6 further support to complete the assignment of Glc_{II}.

Two of the remaining unassigned glucose moieties were assigned as substituents at C-2 and C-3 of Glc_{II} on the basis of HMBC correlations. The anomeric proton observed at δ_H 5.57 showed a HMBC correlation to Glc_{II} C-2 and was assigned as the anomeric proton of Glc_{III}. The anomeric proton observed at δ_H 5.34 showed a HMBC correlation to Glc_{II} C-3 and was assigned as the anomeric proton of Glc_{IV}. The reciprocal HMBC correlations from Glc_{II} H-2 to the anomeric carbon of Glc_{III} and from Glc_{II} H-3 to the anomeric carbon of Glc_{IV} were also observed.

The anomeric proton of Glc_{III} (δ_H 5.57) showed a COSY correlation with a proton at δ_H 4.19 which was assigned as Glc_{III} H-2. Due to data overlap, the COSY spectrum did not allow assignment of H-3 to H-6 protons. Therefore, a series of 1D-TOCSY experiments were performed using selective irradiation of the Glc_{III} anomeric proton with several different mixing times. In addition to confirming the assignments for Glc_{III} H-2, the 1D-TOCSY data showed protons at δ_H 4.24 (Glc_{III} H-3), δ_H 4.27 (Glc_{III} H-4), and δ_H 3.94 (Glc_{III} H-5). Once H-4 was assigned using 1D-TOCSY data, COSY correlations from H-4 to H-5 and in turn to H-6 were used to assign H-6. In the COSY spectrum, Glc_{III} H-4 showed a correlation to Glc_{III} H-5, which in turn showed COSY correlations to δ_H 4.41 and 4.50 of Glc_{III} H-6a and H-6b, respectively. The ^{13}C chemical shifts for Glc_{III} C-2 (δ_C 76.8), C-3 (δ_C 78.9), C-4 (δ_C 72.4), C-5 (δ_C 78.8), and C-6 (δ_C 63.5) were then determined using the ^1H-^{13}C HSQC-DEPT correlations to complete the assignment of Glc_{III}.

The anomeric proton of Glc_{IV} (δ_H 5.34) showed a COSY correlation with a proton at δ_H 4.06 which was assigned as Glc_{IV} H-2. Due to data overlap, the COSY spectrum did not allow assignment of H-3 to H-6 protons. Therefore, a series of 1D-TOCSY

experiments were performed using selective irradiation of the Glc_{IV} anomeric proton with several different mixing times. In addition to confirming the assignments for Glc_{IV} H-2, the 1D-TOCSY data showed protons at δ_H 4.22 (Glc_{IV} H-3), δ_H 4.18 (Glc_{IV} H-4), and δ_H 4.10 (Glc_{IV} H-5). Once H-4 was assigned using 1D-TOCSY data, COSY correlations from H-4 to H-5 and in turn to H-6 were used to assign H-6. In the COSY spectrum, Glc_{IV} H-4 showed a correlation to Glc_{IV} H-5, which in turn showed COSY correlations to δ_H 4.32 and 4.58, Glc_{IV} H-6a and H-6b, respectively. The ^{13}C chemical shifts for Glc_{IV} C-2 (δ_C 75.8), C-3 (δ_C 78.9), C-4 (δ_C 72.0), C-5 (δ_C 79.3), and C-6 (δ_C 62.9) were then determined using the ^1H-^{13}C HSQC-DEPT correlations to complete the assignment of Glc_{IV}.

The large coupling constants observed for the anomeric protons of the glucose moieties at δ_H 6.02 (d, J = 8.1 Hz), 5.57 (d, J = 7.6 Hz), 5.34 (d, J = 7.9 Hz) and δ_H 5.04 (d, J = 8.1 Hz), suggested their β-orientation (Figures 14, 27, and 28). While the remaining anomeric proton at δ_H 5.07 was obscured by the solvent resonance (HDO) it's coupling constant (J = ~8 Hz) evident from 1D TOCSY data (Figure 24) also indicated β-orientation.

A summary of the ^1H and ^{13}C chemical shifts for the glycoside at C-13 are shown in the table below:

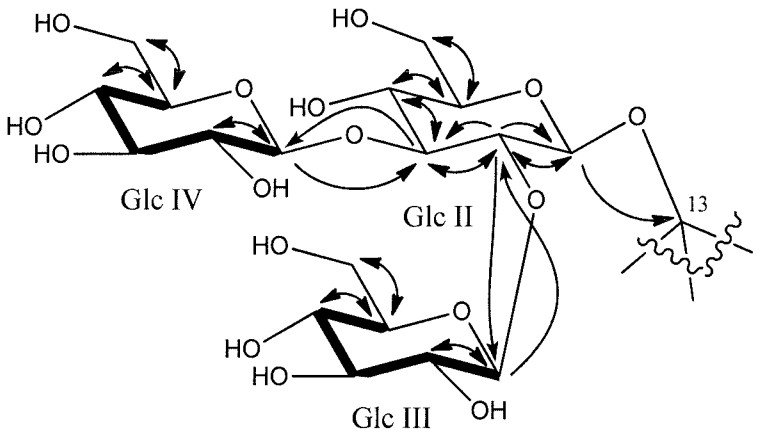
^{1}H and ^{13}C NMR (500 and 125 MHz, pyridine-d_5), Assignments of the Reb *D2* C-13 glycoside.

	Reb *D2*	
Position	^{13}C	^{1}H
Glc_{II}-1	98.7	5.07 (~8)*
Glc_{II}-2	81.3	4.37 m
Glc_{II}-3	88.4	4.18 m
Glc_{II}-4	71.1	3.88 m
Glc_{II}-5	77.9	3.79 m
Glc_{II}-6	63.2	4.08 m 4.47 m
Glc_{III}-1	105.3	5.57 d (7.6)
Glc_{III}-2	76.8	4.19 m
Glc_{III}-3	78.9	4.24 m
Glc_{III}-4	72.4	4.27 m
Glc_{III}-5	78.8	3.94 m
Glc_{III}-6	63.5	4.41 m 4.50m
Glc_{IV}-1	105.3	5.34 d (7.9)
Glc_{IV}-2	75.8	4.06 m
Glc_{IV}-3	78.9[#]	4.22 m[#]
Glc_{IV}-4	72.0	4.18 m
Glc_{IV}-5	79.3	4.10 m
Glc_{IV}-6	62.9	4.32 m 4.58 m

*Anomeric proton was obscured by solvent (HDO) resonance, coupling constant value obtained from 1D-TOCSY data.

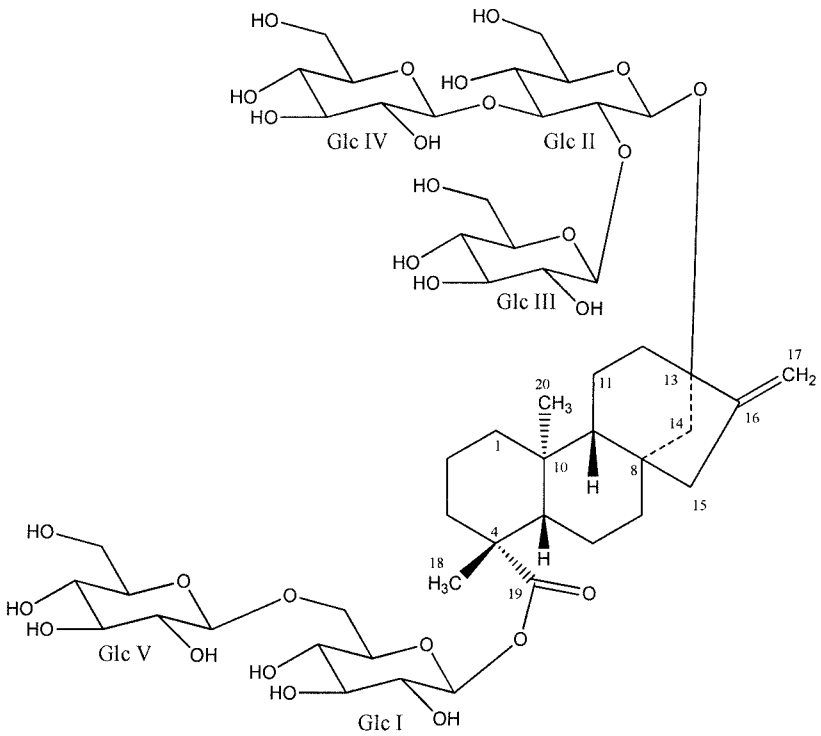
[#]^{1}H and ^{13}C values can be exchangeable between Glc_1-3, Glc_V-3 and Glc_{IV}-3.

A summary of the key HMBC, COSY, and 1D-TOCSY correlations used to assign the C-13 glycoside region are provided below:



= ^{1}H-^{13}C HMBC
= ^{1}H-^{1}H COSY
= ^{1}H-^{1}H spin system based on 1D-TOCSY

NMR and MS analyses allowed a full assignment of structure, shown below. The chemical name of the compound is 13-[(2-*O*-β-D-glucopyranosyl-3-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(6-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl) ester] (rebaudioside *D2* or reb *D2*). The compound is an isomer of rebaudioside D.



EXAMPLE 40

Isolation and Characterization of Reb *M2*

Crude Reaction Sample. The sample, Lot CB-2977-106, used for isolation was prepared according to Example 22 with UGTSL (GI #460409128).

HPLC Analysis. Preliminary HPLC analyses was performed using a Waters 2695 Alliance System with the following method: Phenomenex Synergi Hydro-RP, 4.6 × 250 mm, 4 µm (p/n 00G-4375-E0); Column Temp: 55 °C; Mobile Phase A: 0.0284% NH_4OAc and 0.0116% HOAc in water; Mobile Phase B: Acetonitrile (MeCN); Flow Rate: 1.0 mL/min; Injection volume: 10 µL. Detection was by UV (210 nm) and CAD.

Gradient:

Time (min)	%A	%B
0.0 – 5.0	100	0
20	20	80
25	20	80
30	100	0

Analyses of semi-preparative purification fractions were performed with the following method: Waters Atlantis dC18, 4.6 × 100 mm, 5 µm (p/n 186001340); Mobile Phase A: 25% MeCN in water; Mobile Phase B: 30% MeCN in water; Flow Rate: 1.0 mL/min; Injection volume: 10 µL. Detection was by CAD.

Gradient:

Time (min)	%A	%B
0.0 – 8.5	75	25
10.0	71	29
16.5	70	30
18.5 – 24.5	66	34
26.5 – 29.0	48	52
31 – 37	30	70
38	75	25

LC-MS. Preliminary analysis of the semi-synthetic steviol glycoside mixture was carried out on a Waters AutoPurification HPLC/MS System with a Waters 3100 Mass Detector operating in negative ion mode. Analysis of the sample was performed using the following method: Phenomenex Synergi Hydro-RP, 4.6 × 250 mm, 4 µm (p/n 00G-4375-E0); Column Temp: 55 °C; Mobile Phase A: 0.0284% NH_4OAc and 0.0116% HOAc in water; Mobile Phase B: MeCN; Flow Rate: 1.0 mL/min; Injection volume: 10 µL. Detection was by UV (210 nm), and MSD (-ESI *m/z* 500 – 2000). Gradient conditions were as listed above.

Isolation by HPLC. The purification was performed in two steps. The first method used for the semi-preparative purification is summarized below. Column: Waters Atlantis dC18, 30 × 100 mm, 5 µm (p/n 186001375); Mobile Phase A: 25% MeCN in water; Mobile Phase B: 30% MeCN in water; Flow Rate: 45 mL/min; Injection load: 160 mg dissolved in 20 mL of water. Detection was by UV (205 nm).

Gradient:

Time (min)	%A	%B

0.0 – 5.0	100	0
20	20	80
25	20	80
30	100	0

The secondary purification used the same column and conditions, but isocratic mobile phase: 20% MeCN in water.

MS and MS/MS. MS and MS/MS data were generated with a Waters QTof Premier mass spectrometer equipped with an electrospray ionization source. Samples were analyzed by negative ESI. Samples were diluted with H_2O:MeCN (1:1) by 50 fold and introduced via infusion using the onboard syringe pump. The samples were diluted to yield good s/n which occurred at an approximate concentration of 0.01 mg/mL.

NMR. The sample was prepared by dissolving ~1.0 mg in 150 μL of D_2O and NMR data were acquired on a Bruker Avance 500 MHz instrument with a 2.5 mm inverse detection probe. The ^1H NMR and ^{13}C NMR spectra were referenced to the residual solvent signal HDO (δ_H 4.79 ppm) and TSP (δ_C 0.00 ppm), respectively.

Results and Discussion

Isolation and Purification. Isolation was performed using on a steviol glycoside mixture, Lot number CB-2977-106, prepared according to Example 22 with UGTSL (GI #460409128). The material was analyzed by LC-MS using the method described above (Figure 11). The targeted peak of interest was that at 4.1 min in the TIC chromatogram. The mass spectrum of this peak provided a $[M-H]^-$ ion at *m/z* 1289.7. The provided sample was preliminarily processed in a single injection (160 mg) using the first method condition provided above. This method fractionated the material into 'polar' and 'non-polar' mixtures of glycosides. The 'polar' mixture was then reprocessed using the second-step conditions provided above. The semi-preparative HPLC trace is shown in Figure 12. From this semi-preparative collection, the peak was isolated with a purity >99% (CAD, AUC). The fraction analysis is provided in Figure 13. Following the purification, the fractions were concentrated by rotary evaporation at 35 °C and lyophilized. Approximately 1 mg was obtained.

Mass Spectrometry. The ESI- TOF mass spectrum acquired by infusing a sample of CC-00300 showed a $[M-H]^-$ ion at *m/z* 1289.5266. The mass of the $[M-H]^-$ ion was in good agreement with the molecular formula $C_{56}H_{90}O_{33}$ (calcd for $C_{56}H_{89}O_{33}$: 1289.5286, error: -1.6 ppm) expected for reb *M2*. The MS data confirmed that CC-00300 has a nominal mass of 1290 Daltons with the molecular formula, $C_{56}H_{90}O_{33}$.

The MS/MS spectrum (selecting the $[M-H]^-$ ion at *m/z* 1289.5 for fragmentation) indicated the loss of three glucose units at *m/z* 803.3688 and sequential loss of three glucose moieties at *m/z* 641.3165, 479.2633 and 317.2082.

NMR Spectroscopy. A series of NMR experiments including ^1H NMR (Figure 29), ^{13}C NMR (Figure 30 and 31), ^1H-^1H COSY (Figure 32), HSQC-DEPT (Figure 33), HMBC (Figures 34 and 35), and 1D-TOCSY (Figure 36-39) were performed to allow assignment of reb *M2*.

The ^1H, ^1H-^1H COSY, ^1H-^{13}C HSQC-DEPT and ^1H-^{13}C HMBC NMR data indicated that the central core of the glycoside is a diterpene. The presence of six anomeric protons observed in the ^1H and ^1H-^{13}C HSQC-DEPT spectra confirm six sugar units in the structure. The methylene ^{13}C resonance at δ_C 70.9 in the ^1H-^{13}C HSQC-DEPT spectrum indicated the presence of a 1→6 sugar linkage in the structure. The linkages of sugar units were assigned using ^1H-^{13}C HMBC and 1D-TOCSY correlations.

A HMBC correlation from the methyl protons at δ_H 1.29 to the carbonyl at δ_C 181.5 allowed assignment of one of the tertiary methyl groups (C-18) as well as C-19 and provided a starting point for the assignment of the rest of the aglycone. Additional HMBC correlations from the methyl protons (H-18) to carbons at δ_C 39.8, 43.7, and 59.2 allowed assignment of C3, C4, and C5. Analysis of the ^1H-^{13}C HSQC-DEPT data indicated that the carbon at δ_C 39.8 was a methylene group and the carbon at δ_C 59.2 was a methine which were assigned as C-3 and C-5, respectively. This left the carbon at δ_C 43.7, which did not show a correlation in the HSQC-DEPT spectrum, to be assigned as the quaternary carbon, C-4. The ^1H chemical shifts for C-3 (δ_H 1.16 and 2.28) and C-5 (δ_H 1.24) were assigned using the HSQC-DEPT data. A COSY correlation between one of the H-3 protons (δ_H 1.16) and a proton at δ_H 1.49 allowed assignment of one of the H-2 protons which in turn showed a correlation with a proton at δ_H 0.92 which was assigned to C-1.

The remaining ^{1}H and ^{13}C chemical shifts for C-1 and C-2 were then assigned on the basis of additional COSY and HSQC-DEPT correlations and are summarized in the table below.

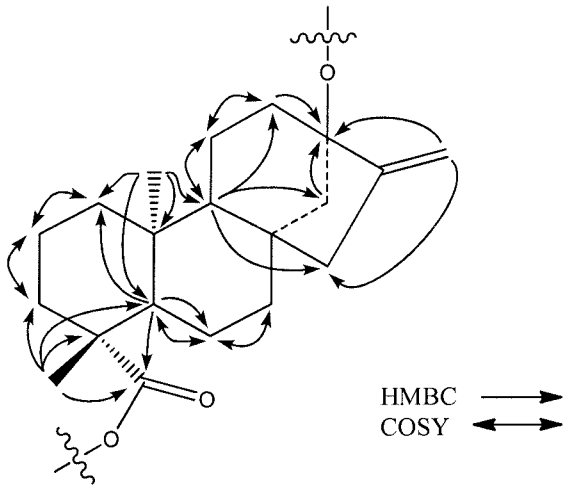
^{1}H NMR (500 MHz, D_2O) and ^{13}C NMR (125 MHz, D_2O/TSP) Assignments of the Reb *M2* aglycone.

Position	^{13}C	^{1}H
1	41.9	0.92 m 1.93 m
2	21.8	1.49 m 1.86 m
3	39.8	1.16 m 2.28 d (13.4)
4	43.7	---
5	59.2	1.24 d (12.1)
6	24.4	1.73 m 1.94 m
7	44.2	1.49 m 1.56 m
8	46.9	---
9	55.5	1.09 d (7.7)
10	42.4	---
11	22.6	1.66 m 1.70 m
12	39.9	1.60 m 2.00 m
13	90.9	---
14	46.9	1.53 d (12.6) 2.21 d (13.6)
15	49.4	2.15 d (17.2) 2.18 d (18.1)
16	164.0	---

17	107.0	4.98 s 5.16 s
18	31.0	1.29 s
19	181.5	---
20	19.1	0.92 s

The other tertiary methyl singlet, observed at δ_H 0.92 showed HMBC correlations to C-1 and C-5 and was assigned as C-20. The methyl protons showed additional HMBC correlations to a quaternary carbon (δ_C 42.4) and a methine (δ_C 55.5) which were assigned as C-10 and C-9, respectively. COSY correlations between H-5 (δ_H 1.24) and protons at δ_H 1.73 and 1.94 then allowed assignment of the H-6 protons which in turn showed correlations to protons at δ_H 1.49 and 1.56 which were assigned to C-7. The ^{13}C chemical shifts for C-6 (δ_C 24.4) and C-7 (δ_C 44.2) were then determined from the HSQC-DEPT data. COSY correlations between H-9 (δ_H 1.09) and protons at δ_H 1.66 and 1.70 allowed assignment of the H-11 protons which in turn showed COSY correlations to protons at δ_H 1.60 and 2.00 which were assigned as the H-12 protons. The HSQC-DEPT data was then used to assign C-11 (δ_C 22.6) and C-12 (δ_C 39.9). The olefinic protons observed at δ_H 4.98 and 5.16 showed HMBC correlations to C-13 (δ_C 90.9) and were assigned to C-17 (δ_C 107.0 via HSQC-DEPT). The olefinic protons H-17 showed HMBC correlations to a carbon at δ_C 49.4 which was assigned as C-15. An additional HMBC correlation from H-9 to a methylene carbon at δ_C 46.9 then allowed assignment of C-14. The ^1H chemical shifts at C-14 (δ_H 1.53 and 2.21) and C-15 (δ_H 2.15 and 2.18) were assigned using the HSQC-DEPT data.

A summary of the key HMBC and COSY correlations used to assign the aglycone region are provided below:



Analysis of the ^{1}H-^{13}C HSQC-DEPT data confirmed the presence of six anomeric protons. Three of the anomeric protons were well resolved at δ_H 5.65 (δ_C 95.5), 4.92 (δ_C 104.9), and 4.50 (δ_C 105.7) in the ^{1}H NMR spectrum. The remaining three anomeric protons observed at δ_H 4.85 (δ_C 98.4), 4.84 (δ_C 105.0), and 4.83 (δ_C 105.3) were overlapped by the residual solvent resonance in the ^{1}H spectrum. The anomeric proton observed at δ_H 5.65 showed a HMBC correlation to C-19 which indicated that it corresponds to the anomeric proton of Glc_I. Similarly, the anomeric proton observed at δ_H 4.85 showed a HMBC correlation to C-13 allowing it to be assigned as the anomeric proton of Glc_{II}.

The Glc_I anomeric proton (δ_H 5.65) showed a COSY correlation to a proton at δ_H 3.96 which was assigned as Glc_I H-2 which in turn showed a COSY correlation to a proton at δ_H 3.89 (Glc_I H-3) which showed a COSY correlation with a proton at δ_H 3.71 (Glc_I H-4). Due to data overlap, the COSY spectrum did not allow assignment of the H-5 or H-6 protons. Therefore, a series of 1D-TOCSY experiments were performed using selective irradiation of the Glc_I anomeric proton with several different mixing times. In addition to confirming the assignments for Glc_I H-2 through H-4, the 1D-TOCSY data showed a proton at δ_H 3.73 assigned as Glc_I H-5 and a proton at δ_H 4.15 assigned as one of the Glc_I H-6 protons. The latter proton was also used for 1D-TOCSY experiments. The selective irradiation of H-6 with several different mixing times also confirmed the assignment of Glc_I H-1 to H-5 as well as the remaining methylene proton of H-6 (δ_H 4.00). Assignment of the ^{13}C chemical shifts for Glc_I C-2 (δ_C 80.5), C-3 (δ_C 79.0), C-4 (δ_C 71.5),

C-5 (δ_C 79.0), and C-6 (δ_C 70.9) was determined using the ^1H-^{13}C HSQC-DEPT data to complete the assignment of Glc_I. Furthermore, the presence of a methylene ^{13}C resonance at δ_C 70.9 in the ^1H-^{13}C HSQC-DEPT spectrum indicated a 1→6 sugar linkage of Glc_I in the structure.

Two of the unassigned glucose moieties were assigned as substituents at C-2 and C-6 of Glc_I on the basis of HMBC correlations. The anomeric proton observed at δ_H 4.83 showed an HMBC correlation to Glc_I C-2 and was assigned as the anomeric proton of Glc_V. The anomeric proton observed at δ_H 4.50 showed a HMBC correlation to Glc_I C-6 and was assigned as the anomeric proton of Glc_{VI}. The reciprocal HMBC correlations from Glc_I H-2 to the anomeric carbon of Glc_V and from Glc_I H-6 to the anomeric carbon of Glc_{VI} were also observed.

The anomeric proton of Glc_V (δ_H 4.83) showed a COSY correlation with a proton at δ_H 3.32 which was assigned as Glc_V H-2. The Glc_V H-2 in turn showed a COSY correlation to a proton at δ_H 3.51 (Glc_V H-3). This latter proton showed an additional correlation with a proton at δ_H 3.38 (Glc_V H-4). H-4 also showed a COSY correlation to a proton at δ_H 3.55 (Glc_V H-5) and Glc_V H-5 in turn showed a COSY correlation to Glc_V H-6 protons (δ_H 3.76 and 3.97). Assignment of the ^{13}C chemical shifts for Glc_V C-2 (δ_C 78.5), C-3 (δ_C 78.7), C-4 (δ_C 72.9), C-5 (δ_C 78.8), and C-6 (δ_C 63.6) was determined using the HSQC-DEPT data. HMBC correlations from Glc_V H-3 to C-2 and C-4 and also from Glc_V H-4 to C-3 and C-6 confirmed the assignments made above to complete the assignment of Glc_V.

Another glucose moiety was assigned as a substituent at C-6 of Glc_I on the basis of ^1H-^{13}C HSQC-DEPT and HMBC correlations. The relatively downfield shift of a methylene ^{13}C resonance of Glc_I at δ_C 70.9 in the HSQC-DEPT spectrum indicated a 1→6 sugar linkage of Glc_I. The anomeric proton observed at δ_H 4.50 showed a HMBC correlation to Glc_I C-6 and was assigned as the anomeric proton of Glc_{VI}. Similarly, methylene protons of Glc_I showed HMBC correlations to the anomeric carbon of Glc_{VI} and this confirmed the presence of a 1→6 sugar linkage between Glc_I and Glc_{VI}. The Glc_{VI} anomeric proton showed a COSY correlation to a proton at δ_H 3.33 which was assigned as Glc_{VI} H-2 which in turn showed a COSY correlation to a proton at δ_H 3.49 (Glc_{VI} H-3). Due to data overlap, the COSY spectrum did not allow assignment of Glc_V H-4 to H-6

based on the COSY correlations. Therefore, a series of 1D-TOCSY experiments were performed using selective irradiation of the Glc_{VI} anomeric proton with different mixing times. In addition to confirming the assignments for Glc_{VI} H-2 through H-3, the 1D-TOCSY data showed protons at δ_H 3.45 (Glc_{VI} H-4) and δ_H 3.48 (Glc_{VI} H-5) and protons at δ_H 3.92 and 3.94 assigned for Glc_{VI} H-6 protons. Assignment of the ^{13}C chemical shifts for Glc_{VI} C-2 (δ_C 78.1), C-3 (δ_C 78.6), C-4 (δ_C 72.3), C-5 (δ_C 78.8), and C-6 (δ_C 64.1) was determined using the ^1H-^{13}C HSQC-DEPT data to complete the assignment of Glc_{VI}.

A summary of the ^1H and ^{13}C chemical shifts for the glycoside at C-19 are found in the table below:

H NMR (500 MHz, D_2O) and ^{13}C NMR (125 MHz, D_2O/TSP) Assignments of the Reb *M2* glycoside.

Position	^{13}C	^1H
Glc_I-1	95.5	5.65 d (7.6)
Glc_I-2	80.5	3.96 m
Glc_I-3	79.0	3.89 m
Glc_I-4	71.5	3.71 m
Glc_I-5	79.0	3.73 m
Glc_I-6	70.9	4.00 m 4.15 d (11.7)
Glc_V-1	105.3 *	4.83* d (8.0)
Glc_V-2	78.5	3.32 m
Glc_V-3	78.7	3.51 m
Glc_V-4	72.9	3.38 m
Glc_V-5	78.8	3.55 m
Glc_V-6	63.6	3.76 m 3.97 m
Glc_{VI}-1	105.7	4.50 d (7.9)
Glc_{VI}-2	78.1	3.33 m
Glc_{VI}-3	78.6	3.49 m

Glc_{VI}-4	72.3	3.45 m
Glc_{VI}-5	78.8	3.48 m
Glc_{VI}-6	64.1	3.92 m 3.94 m

*^{1}H and ^{13}C values can be exchangeable with Glc_{IV}-1 of the following table.

A summary of the key HMBC, COSY, and 1D-TOCSY correlations used to assign the C-19 glycoside region are provided below:

^{1}H NMR (500 MHz, D_2O) and ^{13}C NMR (125 MHz, D_2O/TSP) Assignments of the Reb *M2* glycoside.

Position	$^{13}C^{\#}$	^{1}H
Glc_{II}-1	98.4	4.85 d (7.8)
Glc_{II}-2	81.7	3.75 m
Glc_{II}-3	88.0	3.98 m
Glc_{II}-4	71.3	3.54 m
Glc_{II}-5	80.5	3.96 m
Glc_{II}-6	63.6	3.45 m 3.77 m
Glc_{III}-1	104.9	4.92 d (7.9)
Glc_{III}-2	76.3	3.32 m
Glc_{III}-3	78.8	3.51 m
Glc_{III}-4	73.3	3.26 t (9.5)
Glc_{III}-5	78.8	3.44 m
Glc_{III}-6	64.4	3.75 m 3.94 m
Glc_{IV}-1	105.0	4.84 d (7.8)
Glc_{IV}-2	76.1	3.41 m
Glc_{IV}-3	78.8	3.46 m
Glc_{IV}-4	72.5	3.45 m

Glc_{IV}-5	81.7	3.75 m
Glc_{IV}-6	65.8	3.55 m 3.78 m

Assignment of Glc_{II} was carried out in a similar manner. The Glc_{II} anomeric proton (δ_H 4.85) showed a COSY correlation to a proton at δ_H 3.75 which was assigned as Glc_{II} H-2 which in turn showed a COSY correlation to a proton at δ_H 3.98 (Glc_{II} H-3). This latter proton showed an additional correlation with a proton at δ_H 3.54 (Glc_{II} H-4). H-4 also showed a COSY correlation to a proton at δ_H 3.96 (Glc_{II} H-5). Glc_{II} H-5 also showed a COSY correlation to Glc_{II} H-6 protons (δ_H 3.77 and 3.45). Assignment of the ^{13}C chemical shifts for Glc_{II} C-2 (δ_C 81.7), C-3 (δ_C 88.0), C-4 (δ_C 71.3), C-5 (δ_C 80.5), and C-6 (δ_C 63.6) was determined using the HSQC-DEPT data. HMBC correlations from Glc_{II} H-3 to C-2 and C-4 and also from Glc_{II} H-4 to C-3 and C-6 confirmed the assignments made above to complete the assignment of Glc_{II}.

Two of the remaining unassigned glucose moieties were assigned as substituents at C-2 and C-3 of Glc_{II} on the basis of HMBC correlations. The anomeric proton observed at δ_H 4.92 showed a HMBC correlation to Glc_{II} C-2 and was assigned as the anomeric proton of Glc_{III}. The anomeric proton observed at δ_H 4.84 showed a HMBC correlation to Glc_{II} C-3 and was assigned as the anomeric proton of Glc_{IV}. The reciprocal HMBC correlations between Glc_{II} H-2 and the anomeric carbon of Glc_{III} and between Glc_{II} H-3 and the anomeric carbon of Glc_{IV} were also observed.

The anomeric proton of Glc_{III} (δ_H 4.92) showed a COSY correlation with a proton at δ_H 3.32 which was assigned as Glc_{III} H-2. Due to data overlap, the COSY spectrum did not allow assignment of H-3 to H-6 protons. Therefore, a series of 1D-TOCSY experiments were performed using selective irradiation of the Glc_{III} anomeric proton with different mixing times. In addition to confirming the assignments for Glc_{III} H-2, the 1D-TOCSY data showed protons at δ_H 3.51 (Glc_{III} H-3), δ_H 3.26 (Glc_{III} H-4), and δ_H 3.44 (Glc_{III} H-5). Once H-4 was assigned using 1D-TOCSY data, COSY correlations from H-4 to H-5 and in turn to H-6 were used to assign H-6. In the COSY spectrum, Glc_{III} H-4 showed a correlation to Glc_{III} H-5, which in turn showed COSY correlations to δ_H 3.94

and 3.75 of Glc_{III} H-6a and H-6b, respectively. The ^{13}C chemical shifts for Glc_{III} C-2 (δ_C 76.3), C-3 (δ_C 78.8), C-4 (δ_C 73.3), C-5 (δ_C 78.8), and C-6 (δ_C 64.4) were then determined using the ^1H-^{13}C HSQC-DEPT correlations to complete the assignment of Glc_{III}.

The anomeric proton of Glc_{IV} (δ_H 4.84) which showed a COSY correlation to a proton at δ_H 3.41 was assigned as Glc_{IV} H-2 which in turn showed a COSY correlation to a proton at δ_H 3.46 (Glc_{IV} H-3). This latter proton showed an additional correlation with a proton at δ_H 3.45 (Glc_{IV} H-4) which also showed a COSY correlation to a proton at δ_H 3.75 (Glc_{IV} H-5). Glc_{IV} H-5 also showed a COSY correlation to Glc_{IV} H-6 protons (δ_H 3.55 and 3.78). Assignment of the ^{13}C chemical shifts for Glc_{IV} C-2 (δ_C 76.1), C-3 (δ_C 78.8), C-4 (δ_C 72.5), C-5 (δ_C 81.7), and C-6 (δ_C 65.8) was determined using the HSQC-DEPT data. HMBC correlations from Glc_{IV} H-3 to C-4 and C-5 and also from Glc_{IV} H-4 to C-3 and C-6 confirmed the assignments made above to complete the assignment of Glc_{IV}.

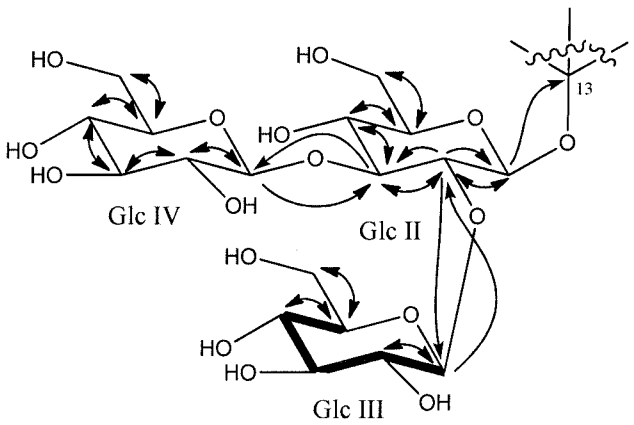
A summary of the ^1H and ^{13}C chemical shifts for the glycoside at C-13 are found in the following table:

^1H NMR (500 MHz, D_2O) and ^{13}C NMR (125 MHz, D_2O/TSP) Assignments of the Reb *M2* glycoside.

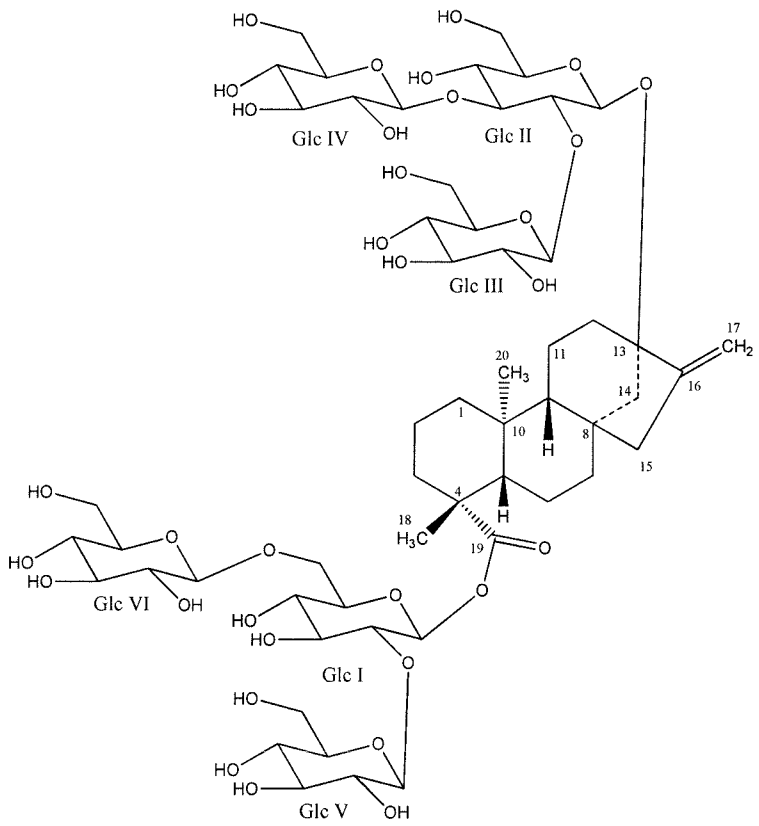
Position	$^{13}C^{\#}$	^1H
Glc_{II}-1	98.4	4.85 d (7.8)
Glc_{II}-2	81.7	3.75 m
Glc_{II}-3	88.0	3.98 m
Glc_{II}-4	71.3	3.54 m
Glc_{II}-5	80.5	3.96 m
Glc_{II}-6	63.6	3.45 m 3.77 m
Glc_{III}-1	104.9	4.92 d (7.9)
Glc_{III}-2	76.3	3.32 m
Glc_{III}-3	78.8	3.51 m

Glc_{III}-4	73.3	3.26 t (9.5)
Glc_{III}-5	78.8	3.44 m
Glc_{III}-6	64.4	3.75 m 3.94 m
Glc_{IV}-1	105.0	4.84 d (7.8)
Glc_{IV}-2	76.1	3.41 m
Glc_{IV}-3	78.8	3.46 m
Glc_{IV}-4	72.5	3.45 m
Glc_{IV}-5	81.7	3.75 m
Glc_{IV}-6	65.8	3.55 m 3.78 m

A summary of the key HMBC, COSY, and 1D-TOCSY correlations used to assign the C-13 glycoside region are provided below:



NMR and MS analyses allowed a full assignment of its structure, shown below. The chemical name of the compound is 13-[(2-*O*-β-D-glucopyranosyl-3-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl)oxy] *ent*-kaur-16-en-19-oic acid-[(2-*O*- β -D-glucopyranosyl-6-*O*- β -D-glucopyranosyl- β -D-glucopyranosyl) ester] (rebaudioside *M2* or reb *M2*). The compound is an isomer of rebaudioside M.



EXAMPLE 41

Directed evolution of UGT76G1 for the conversion of Rebaudioside D to Rebaudioside M(Round 2)

The most active clone from the first round of directed evolution of UGT76G1 (see EXAMPLE 26 UGT76G1var94 containing mutations: Q266E_P272A_R334K_G348P_L379G) was chosen as baseline clone for round 2. A list of 53 mutations was established containing different identified positive mutations from the first round and new mutations obtained by DNA2.0 ProteinGPStm strategy. This list of mutations was subsequently used to design 92 variant genes that contained each 3 different mutations. After codon-optimized for expression in *E. coli* the genes were synthesized, subcloned in the pET30a+ plasmid and used for transformation of *E. coli* BL21 (DE3) chemically competent cells. The obtained cells were grown in Petri-dishes on solid LB medium in the presence of Kanamycin. Suitable colonies were selected and allowed to

grow in liquid LB medium in tubes. Glycerol was added to the suspension as cryoprotectant and 400 µL aliquots were stored at -20°C and at -80°C.

These storage aliquots of *E. coli* BL21(DE3) containing the pET30a+_UGT76G1var plasmids were thawed and added to LBGKP medium (20 g/L Luria Broth Lennox; 50 mM PIPES buffer pH 7.00; 50 mM Phosphate buffer pH 7.00; 2.5 g/L glucose and 50 mg/L of Kanamycine). This culture was allowed to shake in a 96 microtiter plate at 30°C for 8 h.

3.95 mL of production medium containing 60 g/L of Overnight Express™ Instant TB medium (Novagen®), 10 g/L of glycerol and 50 mg/L of Kanamycin was inoculated with 50 µL of above described culture. In a 48 deepwell plate the resulting culture was allowed to stir at 20°C. The cultures gave significant growth and a good OD (600 nm) was obtained. After 44 h, the cells were harvested by centrifugation and frozen.

Lysis was performed by addition of Bugbuster® Master mix (Novagen®) to the thawed cells and the lysate was recovered by centrifugation. Activity tests were performed with 100 µL of fresh lysate that was added to a solution of Rebaudioside D (final concentration 0.5 mM), $MgCl_2$ (final concentration 3 mM) and UDP-Glucose (final concentration 2.5 mM) in 50 mM phosphate buffer pH 7.2.

The reaction was allowed to run at 30°C and samples were taken after 2, 4, 7 and 24 h. to determine conversion and initial rate by HPLC (CAD detection) using the analytical method that was described above for the transformation of Rebaudioside D to Rebaudioside M. In parallel the experiments were performed with baseline clone, Round1-Var94. The conversion after 22 h. and initial rate for this baseline clone was defined as 100% and the normalized conversions and initial rates for the round 2 clones are depicted in the following table:

Clone	Mutations*	Normalized conversion Reb D to Reb Mafter 22h.	Normalized initial rate (0-4h)
Round1-Var94	UGT76G1 (Q266E_P272A_R334K_G348P_L379G) baseline clone	100%	100%
Round2-Var1	Round1-Var94 (A213N_P348G_I411V)	70%	86%
Round2-Var2	Round1-Var94 (K303G_I423M_Q425E)	120%	134%
Round2-Var3	Round1-Var94 (V20L_N138K_S147G)	14%	15%
Round2-Var4	Round1-Var94 (I16V_V133A_L299I)	37%	43%
Round2-Var5	Round1-Var94 (S241V_S274G_Q432E)	75%	72%
Round2-Var6	Round1-Var94 (I16V_L139V_I218V)	62%	68%
Round2-Var7	Round1-Var94 (K334R_N409K_Q432E)	104%	92%

Clone	Mutations*	Normalized conversion Reb D to Reb Mafter 22h.	Normalized initial rate (0-4h)
Round2-Var8	Round1-Var94 (I15L_R141T_I407V)	17%	26%
Round2-Var9	Round1-Var94 (R141T_K303G_G379L)	31%	42%
Round2-Var10	Round1-Var94 (I190L_K303G_P348G)	131%	149%
Round2-Var11	Round1-Var94 (E266Q_F314S_N409R)	106%	132%
Round2-Var12	Round1-Var94 (V133A_I295V_K303G)	43%	49%
Round2-Var13	Round1-Var94 (I16V_S241V_N409R)	80%	79%
Round2-Var14	Round1-Var94 (A239V_K334R_G379L)	58%	55%
Round2-Var15	Round1-Var94 (I190L_K393R_V396L)	118%	126%
Round2-Var16	Round1-Var94 (L101F_I295M_K393R)	84%	89%
Round2-Var17	Round1-Var94 (A239V_E266Q_Q425E)	96%	101%
Round2-Var18	Round1-Var94 (V20L_I190L_I423M)	98%	98%
Round2-Var19	Round1-Var94 (V20L_G379L_S456L)	84%	81%
Round2-Var20	Round1-Var94 (K334R_P348G_N409R)	73%	73%
Round2-Var21	Round1-Var94 (E231A_S241V_E449D)	53%	50%
Round2-Var22	Round1-Var94 (K188R_L299I_V394I)	56%	59%
Round2-Var23	Round1-Var94 (E231A_S274G_V394I)	110%	124%
Round2-Var24	Round1-Var94 (S42A_I295V_Q432E)	71%	78%
Round2-Var25	Round1-Var94 (A213N_A272P_K334R)	95%	80%
Round2-Var26	Round1-Var94 (L158Y_S274K_N409K)	80%	50%
Round2-Var27	Round1-Var94 (K188R_I295M_Q425E)	132%	116%
Round2-Var28	Round1-Var94 (I15L_I295M_V394I)	53%	36%
Round2-Var29	Round1-Var94 (V133A_A239V_V394I)	47%	30%
Round2-Var30	Round1-Var94 (L158Y_F314S_K316R)	107%	72%
Round2-Var31	Round1-Var94 (L158Y_A239V_A272P)	54%	30%
Round2-Var32	Round1-Var94 (F46I_D301N_V396L)	109%	101%
Round2-Var33	Round1-Var94 (L101F_I218V_Q432E)	78%	54%
Round2-Var34	Round1-Var94 (I16V_F46I_I295M)	110%	95%
Round2-Var35	Round1-Var94 (A213N_E266S_I407V)	98%	79%
Round2-Var36	Round1-Var94 (A239V_S274K_I295M)	102%	89%
Round2-Var37	Round1-Var94 (A239V_F314S_S450K)	105%	99%
Round2-Var38	Round1-Var94 (L139V_K188R_D301N)	66%	51%
Round2-Var39	Round1-Var94 (I45V_I218V_S274K)	87%	58%
Round2-Var40	Round1-Var94 (S241V_K303G_V394I)	78%	57%
Round2-Var41	Round1-Var94 (R141T_S274G_K334R)	41%	28%
Round2-Var42	Round1-Var94 (V217L_S274G_L299I)	47%	34%
Round2-Var43	Round1-Var94 (S274G_D301N_P348G)	98%	91%
Round2-Var44	Round1-Var94 (E231A_N409R_S450K)	87%	65%
Round2-Var45	Round1-Var94 (R64H_E231A_K316R)	88%	64%
Round2-Var46	Round1-Var94 (V394I_N409K_I411V)	110%	100%
Round2-Var47	Round1-Var94 (I45V_I295M_K303G)	113%	88%
Round2-Var48	Round1-Var94 (L101F_V396L_L398V)	46%	43%
Round2-Var49	Round1-Var94 (N27S_L101F_S447A)	54%	37%

Clone	Mutations*	Normalized conversion Reb D to Reb Mafter 22h.	Normalized initial rate (0-4h)
Round2-Var50	Round1-Var94 (S274G_F314S_L398V)	129%	156%
Round2-Var51	Round1-Var94 (E266Q_L299I_K393R)	70%	51%
Round2-Var52	Round1-Var94 (V217L_E266S_V394I)	62%	48%
Round2-Var53	Round1-Var94 (N138K_A272P_N409R)	118%	102%
Round2-Var54	Round1-Var94 (E266S_F314S_Q432E)	124%	146%
Round2-Var55	Round1-Var94 (D301N_G379L_L398V)	56%	45%
Round2-Var56	Round1-Var94 (F46I_E266S_K334R)	123%	142%
Round2-Var57	Round1-Var94 (A272P_V394I_Q432E)	133%	142%
Round2-Var58	Round1-Var94 (V394I_I407V_S456L)	118%	114%
Round2-Var59	Round1-Var94 (I218V_E266Q_I423M)	106%	98%
Round2-Var60	Round1-Var94 (A272P_G379L_I407V)	80%	63%
Round2-Var61	Round1-Var94 (E231A_K303G_S456L)	113%	110%
Round2-Var62	Round1-Var94 (I190L_E266Q_I407V)	150%	167%
Round2-Var63	Round1-Var94 (N27S_L139V_I295V)	43%	25%
Round2-Var64	Round1-Var94 (V217L_I423M_S447A)	67%	51%
Round2-Var65	Round1-Var94 (L158Y_E266S_E449D)	68%	43%
Round2-Var66	Round1-Var94 (S42A_F46I_I407V)	160%	203%
Round2-Var67	Round1-Var94 (N138K_E231A_D301N)	118%	93%
Round2-Var68	Round1-Var94 (K188R_G379L_N409R)	52%	35%
Round2-Var69	Round1-Var94 (I15L_E231A_V396L)	38%	22%
Round2-Var70	Round1-Var94 (E231A_Q425E_Q432E)	115%	119%
Round2-Var71	Round1-Var94 (D301N_K316R_Q425E)	126%	121%
Round2-Var72	Round1-Var94 (L139V_I295M_F314S)	76%	91%
Round2-Var73	Round1-Var94 (S147G_E266S_D301N)	30%	18%
Round2-Var74	Round1-Var94 (R64H_S147G_S447A)	23%	12%
Round2-Var75	Round1-Var94 (S42A_K303G_L398V)	95%	110%
Round2-Var76	Round1-Var94 (I45V_D301N_E449D)	62%	60%
Round2-Var77	Round1-Var94 (V133A_E266S_I411V)	37%	28%
Round2-Var78	Round1-Var94 (I45V_N409R_Q425E)	63%	59%
Round2-Var79	Round1-Var94 (R141T_A272P_F314S)	23%	10%
Round2-Var80	Round1-Var94 (E266S_S274G_N409R)	81%	91%
Round2-Var81	Round1-Var94 (N409K_Q425E_S450K)	81%	84%
Round2-Var82	Round1-Var94 (N27S_R64H_K393R)	47%	37%
Round2-Var83	Round1-Var94 (S42A_A213N_V217L)	62%	46%
Round2-Var84	Round1-Var94 (N27S_S274K_I407V)	49%	44%
Round2-Var85	Round1-Var94 (I411V_Q425E_S456L)	75%	81%
Round2-Var86	Round1-Var94 (A239V_K316R_E449D)	83%	72%
Round2-Var87	Round1-Var94 (S147G_A239V_P348G)	18%	7%
Round2-Var88	Round1-Var94 (V20L_S274G_S450K)	71%	68%
Round2-Var89	Round1-Var94 (F314S_V394I_S447A)	88%	123%
Round2-Var90	Round1-Var94 (R64H_E266Q_I295M)	45%	47%
Round2-Var91	Round1-Var94 (N138K_I295V_I407V)	50%	51%

Clone	Mutations*	Normalized conversion Reb D to Reb Mafter 22h.	Normalized initial rate (0-4h)
Round2-Var92	Round1-Var94 (I15L_P348G_Q432E)	18%	13%

*Mutations are noted as follows: reference gene-original amino acid-position-new amino acid: For example the mutation of an alanine at position 33 to a glycine for variant 94 from the first round of directed evolution of UGT76G1 is noted as Round1-Var94 (A33G)

Modeling of these results allowed to obtain a ranking of the effect of each mutation. The following mutations were determined as being beneficial for activity: S42A, F46I, I190L, S274G, I295M, K303G, F314S, K316R, K393R, V394I, I407V, N409K, N409R, Q425E, Q432E, S447A, S456L.

EXAMPLE 42

In vivo production of AtSUS

SEQ ID 13

AtSUS
>gi|79328294|ref|NP_001031915.1| sucrose synthase 1 [*Arabidopsis thaliana*]

MANAERMITRVHSQRERLNETLVSERNEVLALLSRVEAKGKGILQQNQIIAEFEAL
PEQTRKKLEGGPFFDLLKSTQEAIVLPPWVALAVRPRPGVWEYLRVNLHALVVEE
LQPAEFLHFKEELVDGVKNGNFTLELDFEPFNASIPRPTLHKYIGNGVDFLNRHLS
AKLFHDKESLLPLLKFLRLHSHQGKNLMLSEKIQNLNTLQHTLRKAEEYLAELKS
ETLYEEFEAKFEEIGLERGWGDNAERVLDMIRLLLDLLEAPDPCTLETFLGRVPMV
FNVVILSPHGYFAQDNVLGYPDTGGQVVYILDQVRALEIEMLQRIKQQGLNIKPRI
LILTRLLPDAVGTTCGERLERVYDSEYCDILRVPFRTEKGIVRKWISRFEVWPYLET
YTEDAAVELSKELNGKPDLIIGNYSDGNLVASLLAHKLGVTQCTIAHALEKTKYP
DSDIYWKKLDDKYHFSCQFTADIFAMNHTDFIITSTFQEIAGSKETVGQYESHTAF
TLPGLYRVVHGIDVFDPKFNIVSPGADMSIYFPYTEEKRRLTKFHSEIEELLYSDVE
NKEHLCVLKDKKKPILFTMARLDRVKNLSGLVEWYGKNTRLRELANLVVVGGD
RRKESKDNEEKAEMKKMYDLIEEYKLNGQFRWISSQMDRVRNGELYRYICDTKG
AFVQPALYEAFGLTVVEAMTCGLPTFATCKGGPAEIIVHGKSGFHIDPYHGDQAA
DTLADFFTKCKEDPSHWDEISKGGLQRIEEKYTWQIYSQRLLTLTGVYGFWKHVS
NLDRLEARRYLEMFYALKYRPLAQAVPLAQDD

The synthetic gene of AtSuS that was codon optimized for expression in *E. coli* and subcloned in the pET30a+ plasmid using the NdeI and XhoI restriction sites. The pET30A+ vector containing the AtSUS gene was used to transform electrocompetent *E. coli* Bl21(DE3) cells. The obtained cells were grown in petri-dishes in the presence of Kanamycin and suitable colonies were selected and allowed to grow in liquid LB medium

(erlenmeyer flasks). Glycerol was added to the suspension as cryoprotectant and 400 µL aliquots were stored at -20°C and at -80°C.

The storage aliquots of *E. coli* BL21(DE3) containing the pET30A+_AtSUS plasmids were thawed and added to 30 mL of LBGKP medium (20 g/L Luria Broth Lennox; 50 mM PIPES buffer pH 7.00; 50 mM Phosphate buffer pH 7.00; 2.5 g/L glucose and 50 mg/L of Kanamycine). This culture was allowed to shake at 135 rpm at 30°C for 8 h.

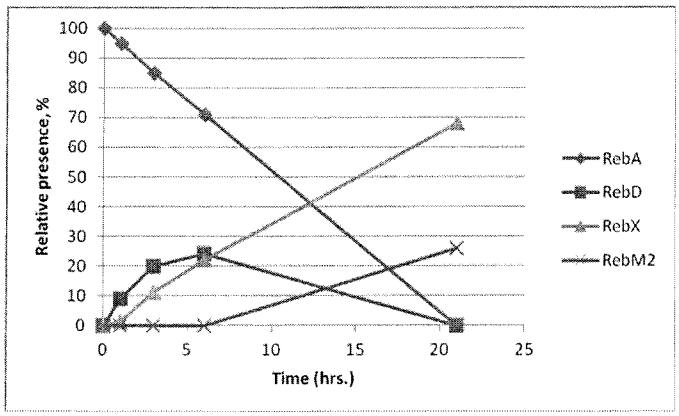
The production medium contained 60 g/L of overnight express instant TB medium (Novagen), 10 g/L of glycerol and 50 mg/L of Kanamycine. The preculture was added to 800 mL of this medium and the solution was allowed to stir at 20°C while taking samples to measure the OD and pH. The culture gave significant growth and a good OD was obtained. After 40 h, the cells were harvested by centrifugation and frozen to obtain 30.1 g of cell wet weight.

Lysis was performed by Fastprep (MP Biomedicals, Lysing matrix B, speed 6.0, 3 x 40 sec) with a cell suspension of 200 mg of cells in 1.0 mL of 50 mM Tris buffer pH 7.5. The lysate was recovered by centrifugation and used fresh.

EXAMPLE 43

Conversion of Rebaudioside A to Rebaudioside M with *in situ* prepared UDP-Glucose using UGTSL2, UGT76G1-R1-F12 and AtSUS

The reaction was performed at 1 mL scale using 100 mM of sucrose, 3 mM of $MgCl_2$, 0.25 mM of UDP and 0.5 mM of Rebaudioside A in potassium phosphate buffer (50 mM final concentration, pH 7.5). The reaction was started by adding 15 µL of UGTSL2 (see EXAMPLE 27) lysate (2 U/mL), 150 µL of UGT76G1var94 (see EXAMPLE 26) (2.5 U/mL) and 15 µL of AtSUS (see EXAMPLE 42) (400 U/mL). The reaction was followed by HPLC after quenching 125 µL samples with 10 µL of 2 N H_2SO_4 and 115 µL of 60% methanol. 68% of Rebaudioside M and 26% of Rebaudioside M2 was obtained after 21 h of reaction time. The results are presented in Figure 69.



Although various embodiments of the present invention have been disclosed in the foregoing description for purposes of illustration, it should be understood that a variety of changes, modifications and substitutions may be incorporated without departing from either the spirit of scope of the present invention.

CLAIMS
We claim:

1. A method for producing highly purified target steviol glycosides, comprising the steps of:

a. providing a starting composition comprising an organic compound with at least one carbon atom;

b. providing a microorganism containing at least one enzyme selected from steviol biosynthesis enzymes, UDP-glycosyltransferases, and optionally UDP-glucose recycling enzymes;

c. contacting the microorganism with a medium containing the starting composition to produce a medium comprising at least one target steviol glycoside.

2. A method for producing highly purified target steviol glycosides, comprising the steps of:

a. providing a starting composition comprising an organic compound with at least one carbon atom;

b. providing a biocatalyst comprising at least one enzyme selected from steviol biosynthesis enzymes, UDP-glycosyltransferases, and optionally UDP-glucose recycling enzymes;

c. contacting the biocatalyst with a medium containing the starting composition to produce a medium comprising at least one target steviol glycoside.

3. The method of claim 1 or 2 further comprising the step of:

d. separating the target steviol glycoside from the medium to provide a highly purified target steviol glycoside composition.

4. The method of claim 1 or 2, wherein the staring composition is selected from the group consisting polyols, carbohydrates, steviol, steviol glycosides and combinations thereof.

5. The method of claim 1, 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 biocatalyst is selected from the group consisting of whole cell suspension, crude lysate or purified enzymes in free or immobilized form.

7. The method of claim 1 or 2, wherein the target steviol glycoside is selected from the group consisting of stevioside, reb *A,* reb *D,* reb *D2*, reb *E,* reb *M,* reb *M2* and mixtures thereof.

8. The method of claim 1 or 2, wherein the target steviol glycoside is produced within the cell or in outside medium and is separated using crystallization, separation by membranes, centrifugation, extraction, chromatographic separation or a combination of such methods.

9. The method of claim 1 or 2, wherein the target steviol glycoside content is greater than about 95% by weight on a dry basis.

10. The method of claim 9, wherein the target steviol glycoside is selected from stevioside, reb *A*, reb *E*, reb *D*, reb *D2*, reb *M*, and reb *M2*.

11. The method of claim 9, wherein the target steviol glycoside is reb *M*.

12. A highly purified target steviol glycoside composition prepared according to the method of claim 1 or 2, wherein the target steviol glycoside content is greater than about 95% by weight on a dry basis.

13. The highly purified target steviol glycoside composition of claim 12, wherein the target steviol glycoside is selected from reb *D* and reb *M*.

14. A highly purified target steviol glycoside composition prepared according to the method of claim 1 or 2, wherein the target steviol glycoside is polymorphic.

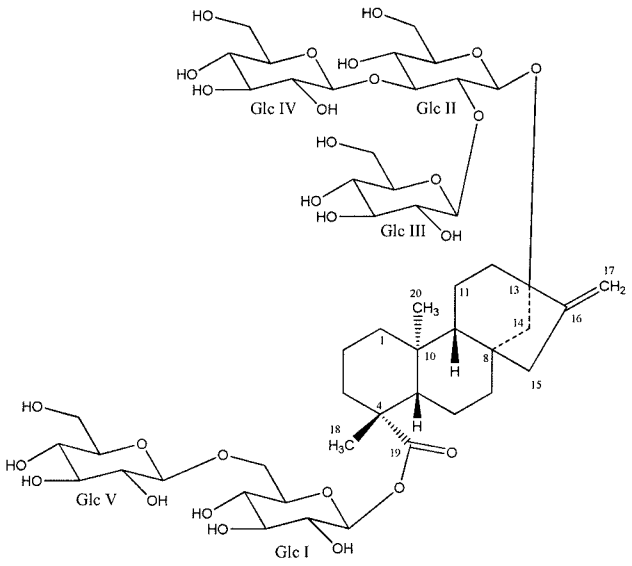
15. The highly purified target steviol glycoside composition of claim 14, wherein the target steviol glycoside is reb *D* or reb *M*.

16. A consumable product comprising the highly purified target glycoside composition of claim 1 or 2, wherein the product is selected from the group consisting of a food, a beverage, a pharmaceutical composition, a tobacco product, a nutraceutical composition, an oral hygiene composition, and a cosmetic composition.

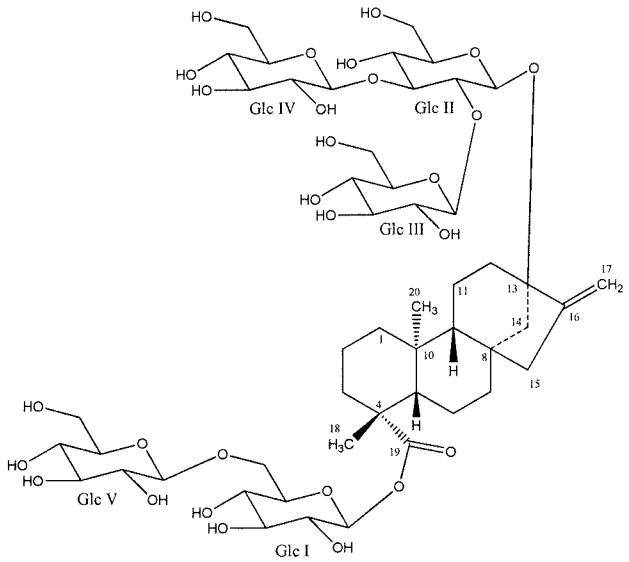
17. A consumable product comprising the highly purified target steviol glycoside composition of claim 1 or 2, wherein the product is selected from the group consisting of a food, a beverage, a pharmaceutical composition, a tobacco product, a nutraceutical composition, an oral hygiene composition, and a cosmetic composition, and wherein the target steviol glycoside is reb *D*.

18. A consumable product comprising the highly purified target steviol glycoside composition of claim 1 or 2, wherein the product is selected from the group consisting of a food, a beverage, a pharmaceutical composition, a tobacco product, a nutraceutical composition, an oral hygiene composition, and a cosmetic composition, and wherein the target steviol glycoside is reb *M*.

19. Isolated and purified reb *D2* having the following structure:



20. Reb *D2* having the following structure:



21. A consumable product comprising reb *D2*.

22. The consumable product of claim 21, wherein the composition is selected from the group consisting of beverages; 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 and medicinal products.

23. A beverage comprising reb *D2*.

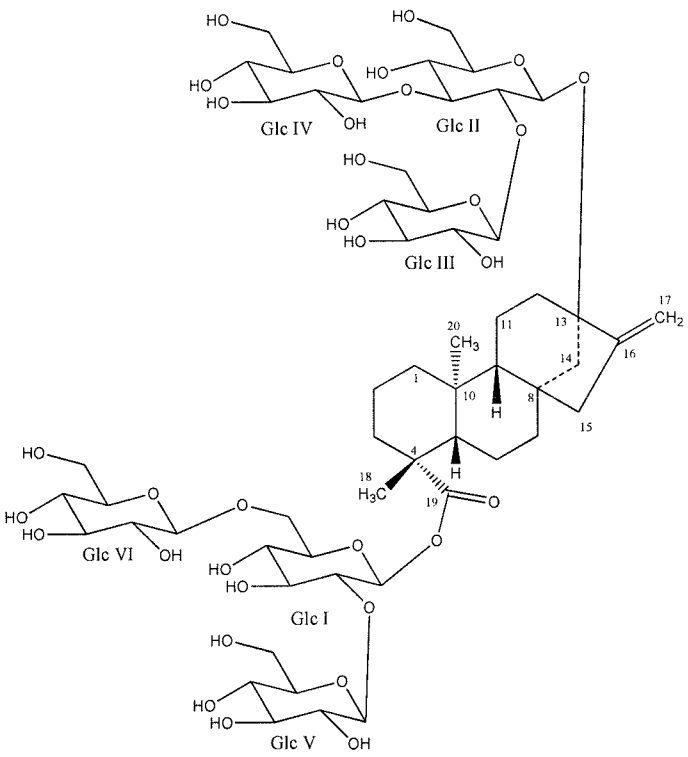
24. A method for preparing reb *D2* comprising:

a. contacting a starting composition comprising reb *A* with an enzyme capable of transforming reb *A* to reb *D2*, UDP-glucose, and optionally UDP-glucose recycling enzymes to produce a composition comprising reb *D2*; and

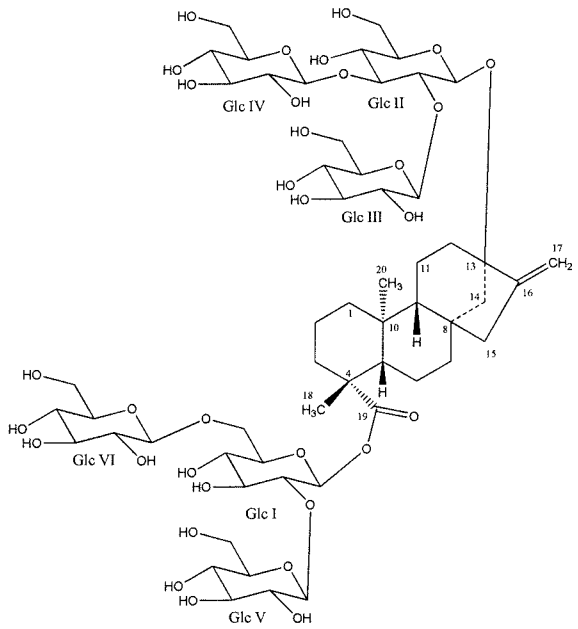
b. isolating a composition comprising reb *D2*.

25. The method of claim 24, further comprising purifying the composition comprising reb *D2* to provide reb *D2* having a purity greater than about 95% by weight on an anhydrous basis.

26. Isolated and purified reb *M2* having the following structure:



27. Reb *M2* having the following structure:



28. A consumable product comprising reb *M2*.

29. The consumable product of claim 28, wherein the composition is selected from the group consisting of beverages; 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 and medicinal products.

30. A beverage comprising reb *M2*.

31. A method for preparing reb *M2* comprising:

a. contacting a starting composition comprising reb *D2* with an enzyme capable of transforming reb *D2* to reb *M2*, UDP-glucose, and optionally UDP-glucose recycling enzymes to produce a composition comprising reb *M2*; and

b. isolating a composition comprising reb *M2*.

32. The method of claim 31, further comprising purifying the composition comprising reb *M2* to provide reb *M2* having a purity greater than about 95% by weight on an anhydrous basis.

33. The consumable product of claim 21, further comprising at least one 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.

34. The consumable product of claim 21, further comprising at least one functional ingredient selected from the group consisting of saponins, antioxidants, dietary fiber sources, fatty acids, vitamins, glucosamine, minerals, preservatives, hydration agents, probiotics, prebiotics, weight management agents, osteoporosis management agents, phytoestrogens, long chain primary aliphatic saturated alcohols, phytosterols and combinations thereof.

35. The consumable product of claim 21, further comprising a compound selected from the group consisting of reb *A*, reb *B*, reb *D*, NSF-02, Mogroside V, Luo Han Guo, allulose, allose, D-tagatose, erythritol and combinations thereof.

36. A tabletop sweetener composition comprising reb *D2*.

37. The consumable product of claim 28, further comprising at least one 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.

38. The consumable product of claim 28, further comprising at least one functional ingredient selected from the group consisting of saponins, antioxidants, dietary fiber sources, fatty acids, vitamins, glucosamine, minerals, preservatives, hydration agents, probiotics, prebiotics, weight management agents, osteoporosis management agents, phytoestrogens, long chain primary aliphatic saturated alcohols, phytosterols and combinations thereof.

39. The consumable product of claim 28, further comprising a compound selected from the group consisting of Reb A, Reb B, Reb D, NSF-02, Mogroside V, Luo Han Guo, allulose, allose, D-tagatose, erythritol and combinations thereof

40. A tabletop sweetener composition comprising reb *M2*.

41. A method for enhancing the sweetness of a beverage comprising a sweetener comprising:

a.) providing a beverage comprising a sweetener; and

b.) adding a sweetness enhancer selected from reb *D2*, reb *M2* or a combination thereof,

wherein reb *D2* and/or reb *M2* is present in a concentration at or below the sweetness recognition threshold.

42. Target steviol glycoside of claim 1 or 2, in combination with at least one naturally occurring high intensity sweetener selected from group including stevia, stevia extract, steviolmonoside, steviolbioside, rubusoside, dulcoside *A*, dulcoside *B*, rebaudioside *B*, rebaudioside *G*, stevioside, rebaudioside *C*, rebaudioside *F*, rebaudioside *A*, rebaudioside *I*, rebaudioside *E*, rebaudioside *H*, rebaudioside *L*, rebaudioside *K*, rebaudioside *J*, rebaudioside *M*, rebaudioside *M2*, rebaudioside *D*, rebaudioside *D2*, rebaudioside *N* or rebaudioside *O*, or other glycoside of steviol found in *Stevia rebaudiana*, 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, mogroside VI, grosmomomside, siamenoside or other glycoside of mogrol found in *Siraitia grosvenorii* and/or combination thereof.

43. The method of claim 1 or 2, wherein the target steviol glycoside content is greater than about 99% by weight on a dry basis.

FIG. 1

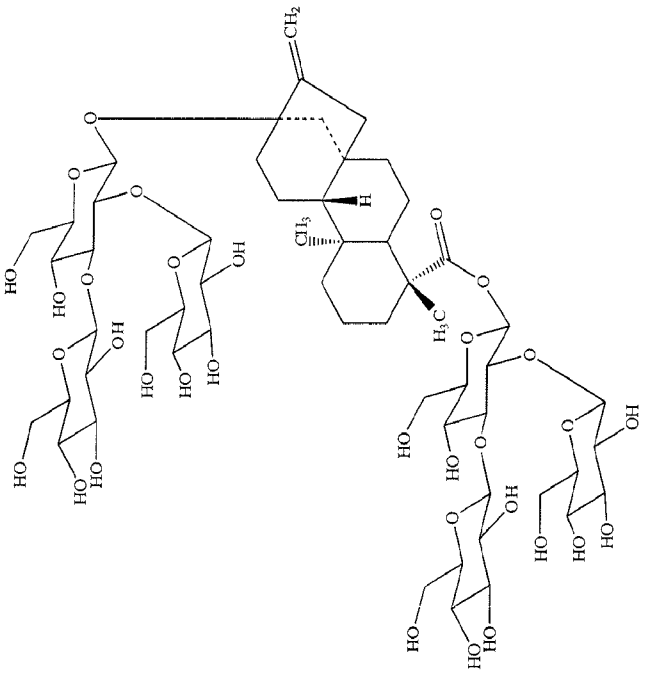


FIG. 2

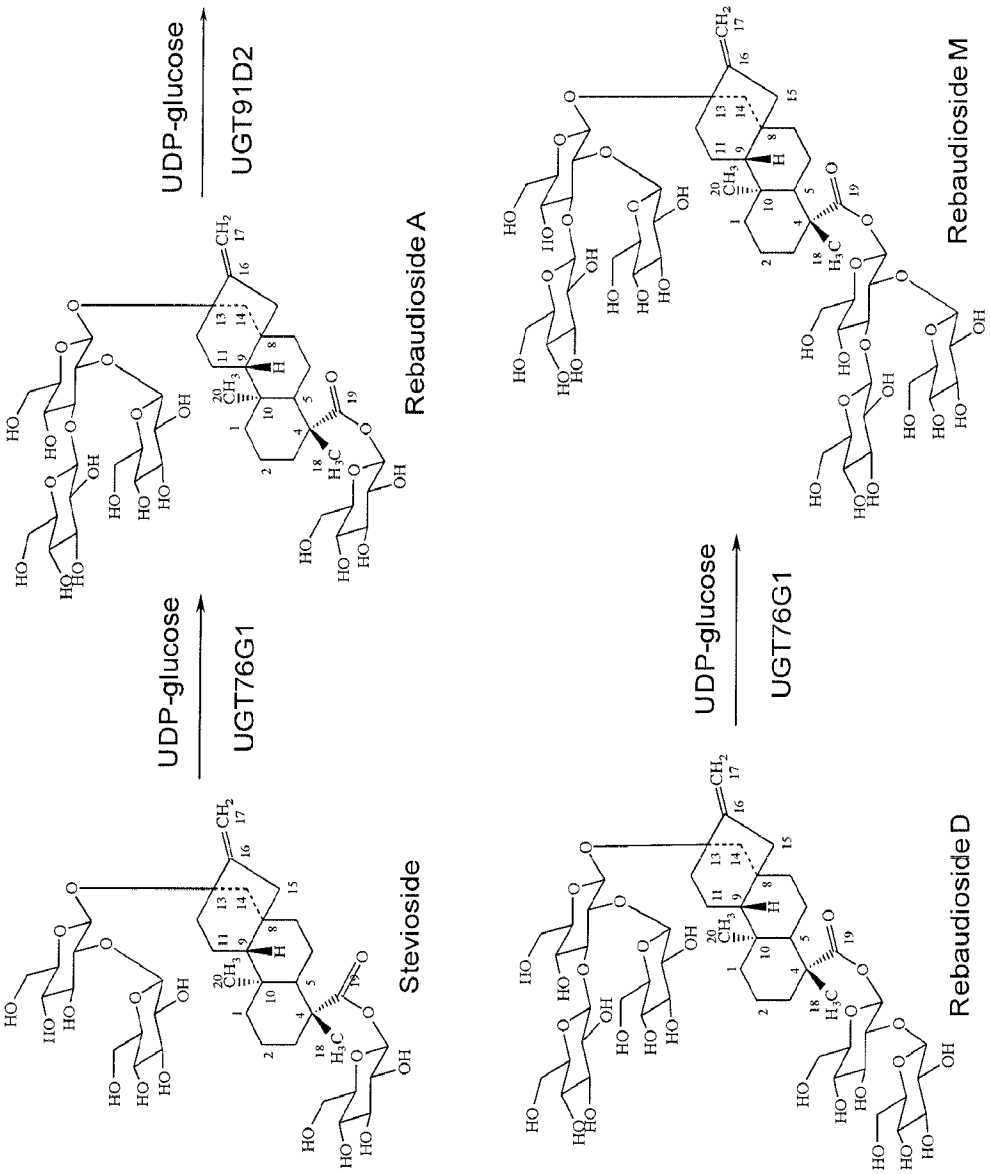

UDP-glucose
UGT91D2
Rebaudioside A
UDP-glucose
UGT76G1
Stevioside
UDP-glucose
UGT76G1
Rebaudioside M
Rebaudioside D

FIG. 3

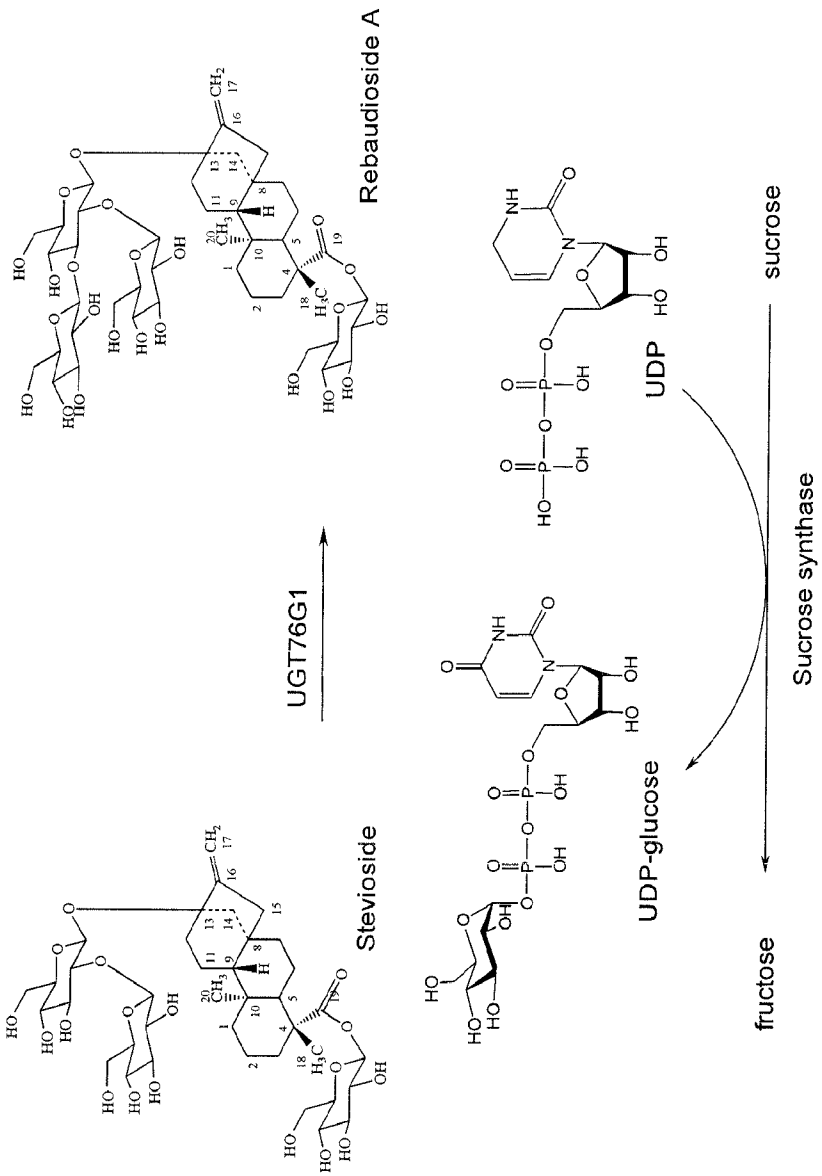

Rebaudioside A
UGT76G1
Stevioside
UDP
UDP-glucose
sucrose
Sucrose synthase
fructose

FIG. 4

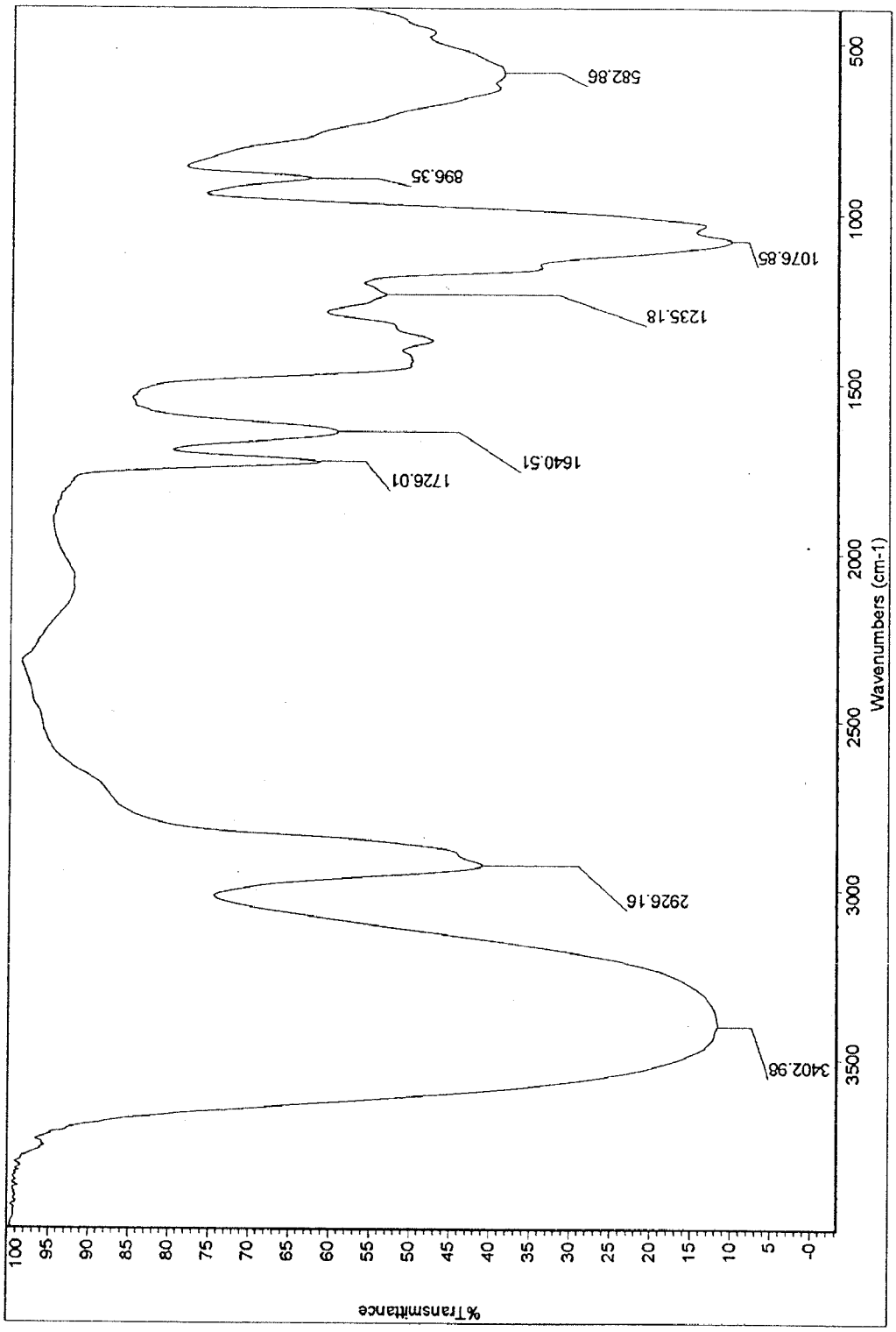

582.86
896.35
1076.85
1235.18
1640.51
1726.01
2926.16
3402.98
%Transmittance
Wavenumbers (cm-1)

FIG. 5

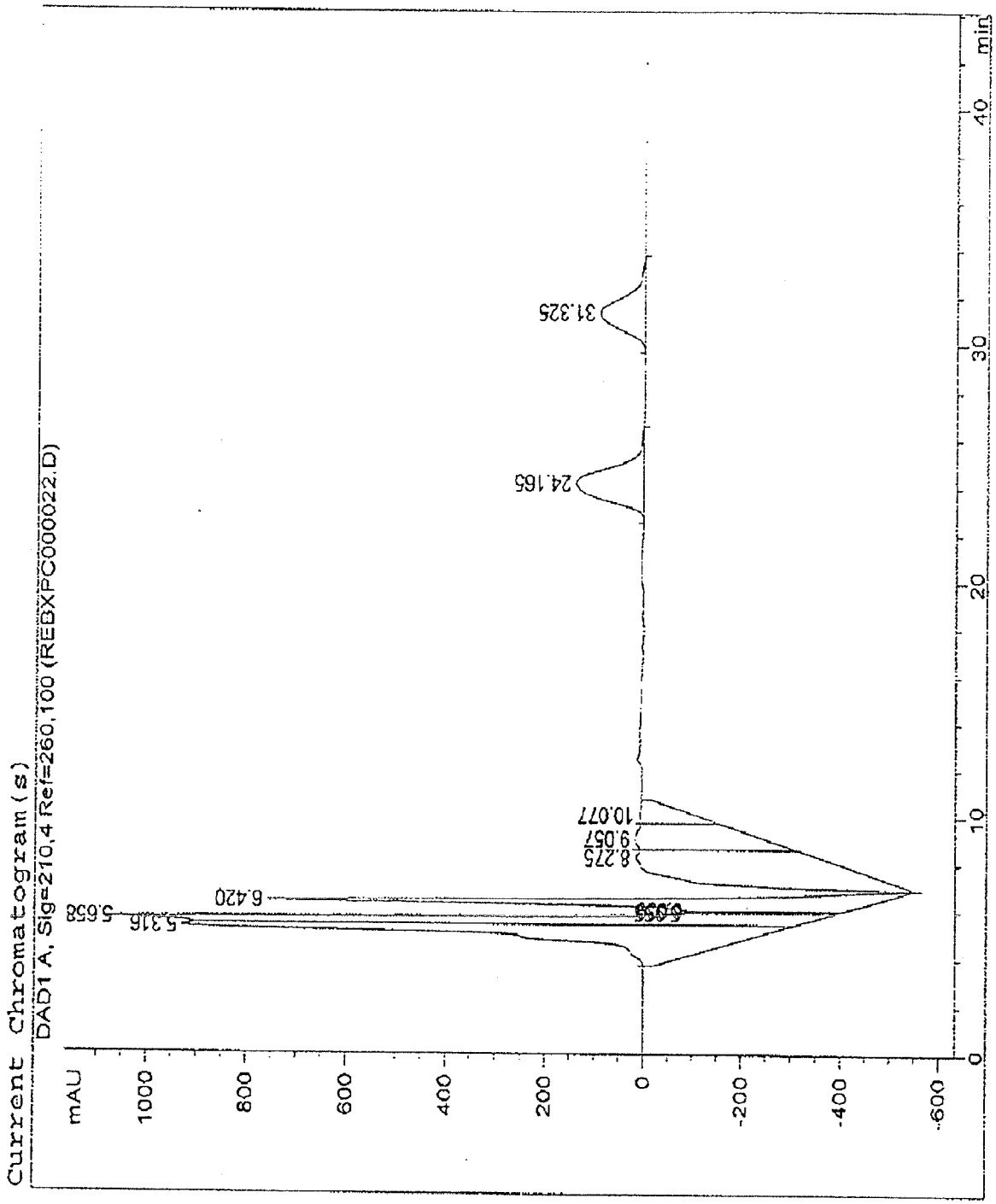

Current Chromatogram (s)
DAD1 A, Sig=210,4 Ref=260,100 (REBXPC000022.D)
mAU
1000
800
600
400
200
0
-200
-400
-600
min
0
10
20
30
40
31.325
24.165
10.077
9.057
8.275
6.420
5.658
5.316

FIG. 6 HPLC Chromatogram of Purified Rebaudioside M from Biotransformation of Rebaudioside D

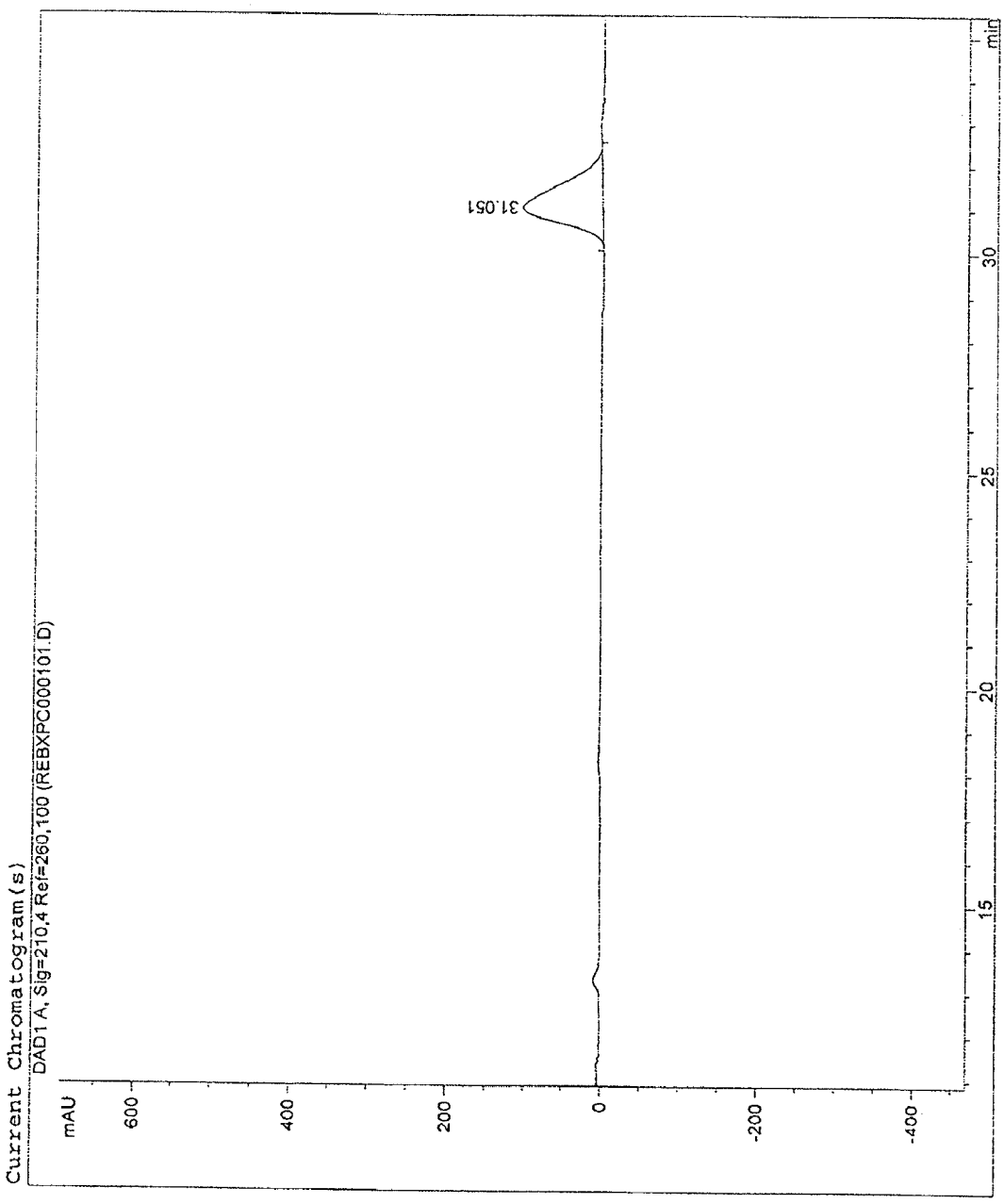


FIG. 7 HPLC Chromatogram of Standard Rebaudioside M

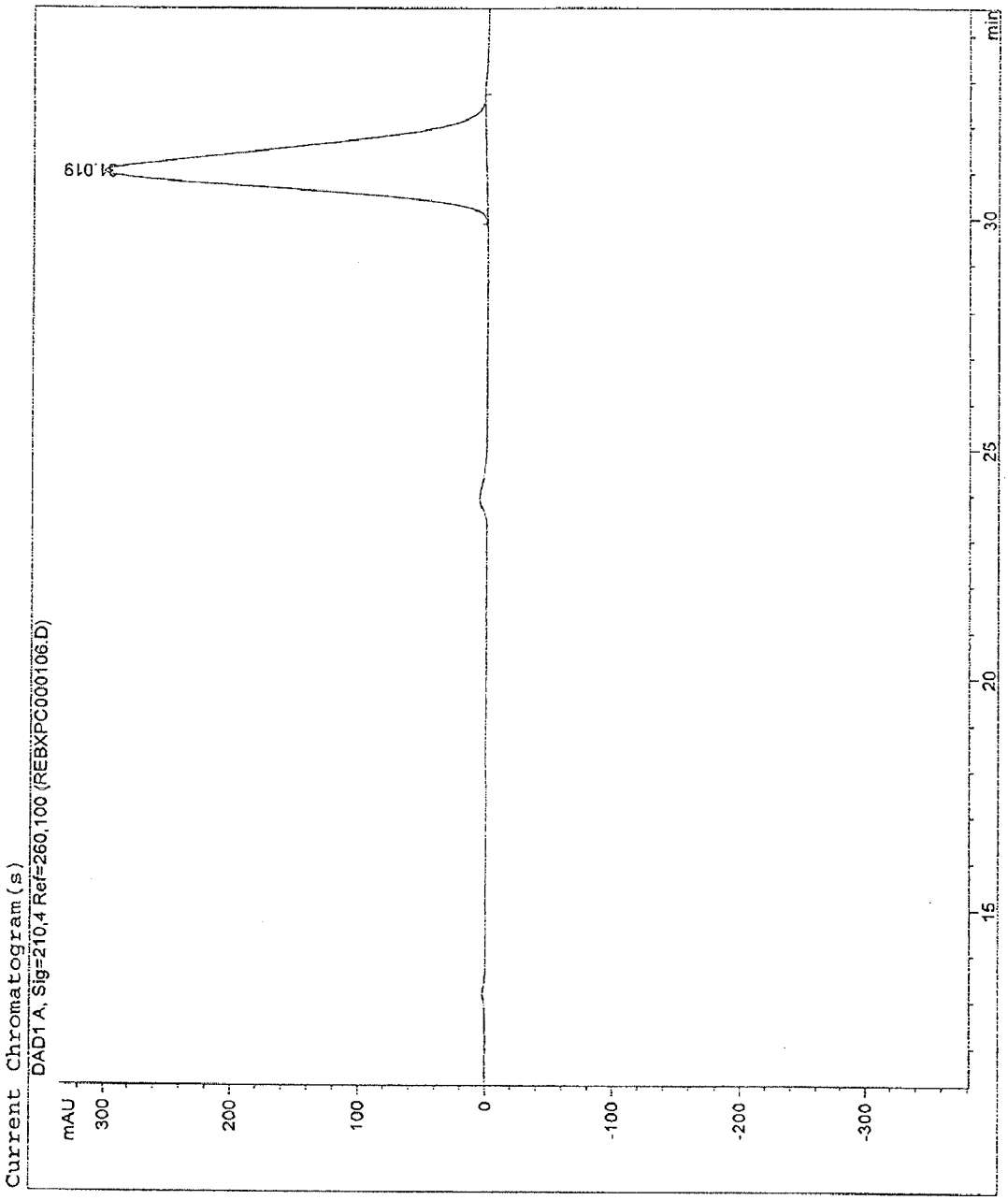


FIG. 8

Co-HPLC Chromatogram of Standard Rebaudioside M and Rebaudioside M Purified from Biotransformation of Rebaudioside D

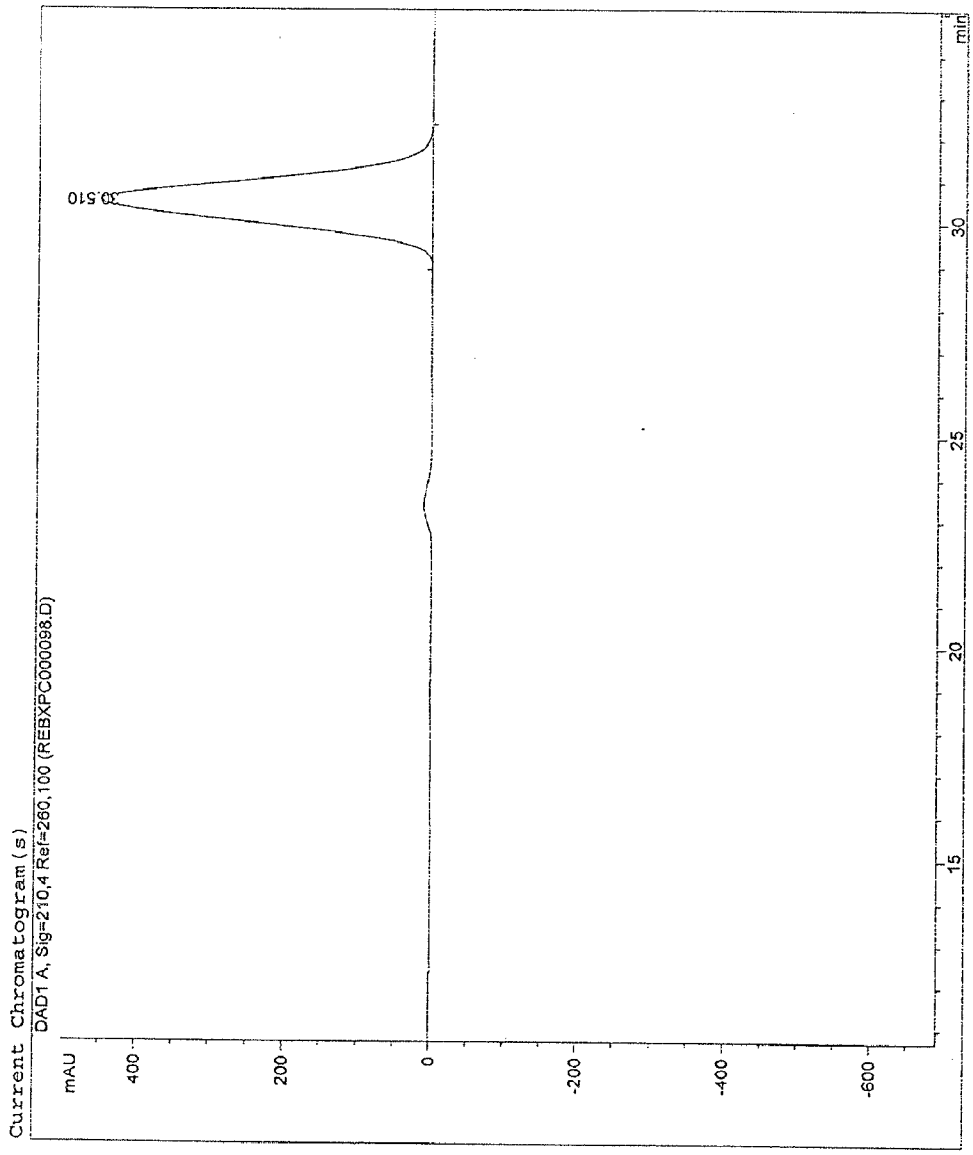


FIG. 9 Overlay of ^{1}H NMR Spectrum of Rebaudioside M Standard and Purified Rebaudioside M from Biotransformation of Rebaudioside D

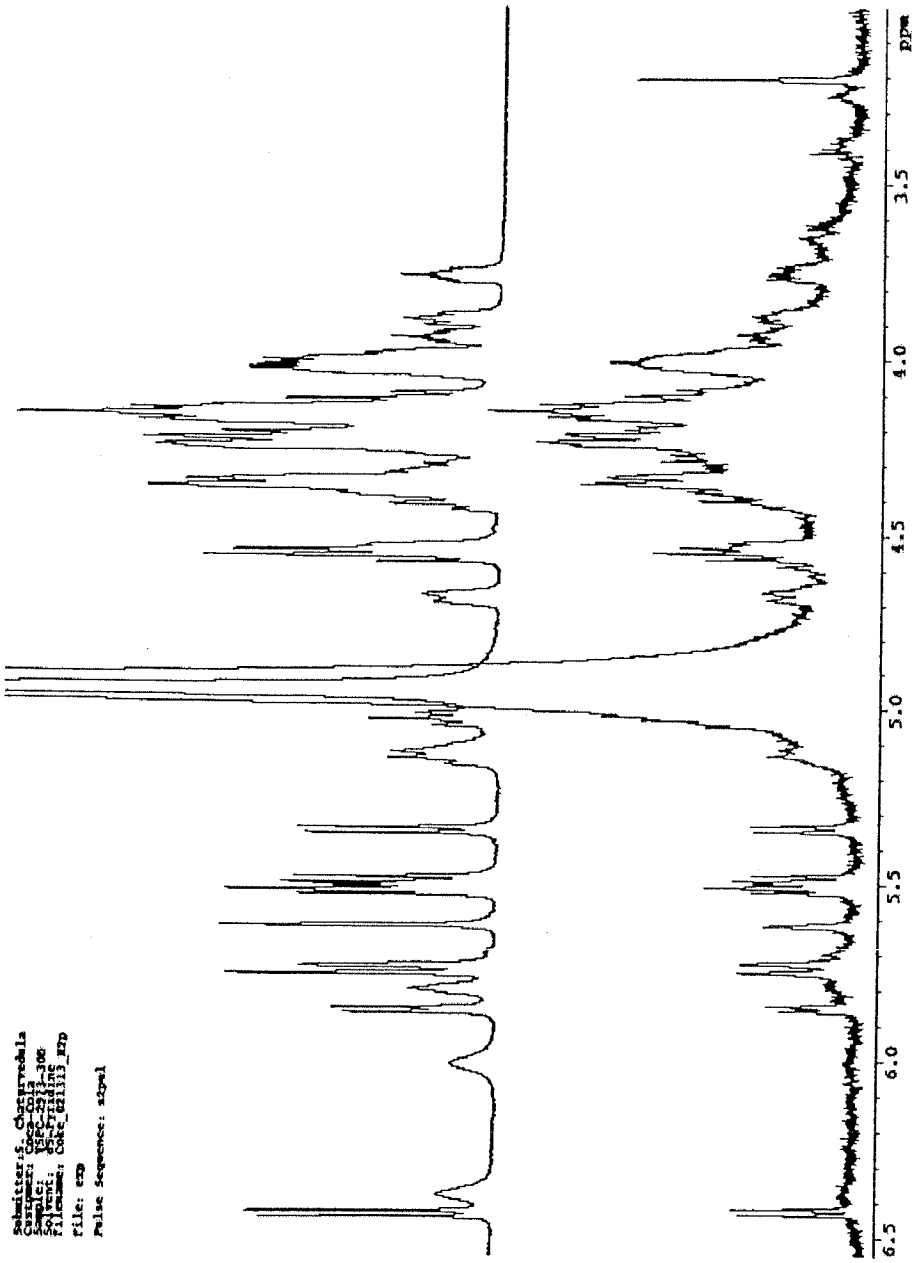
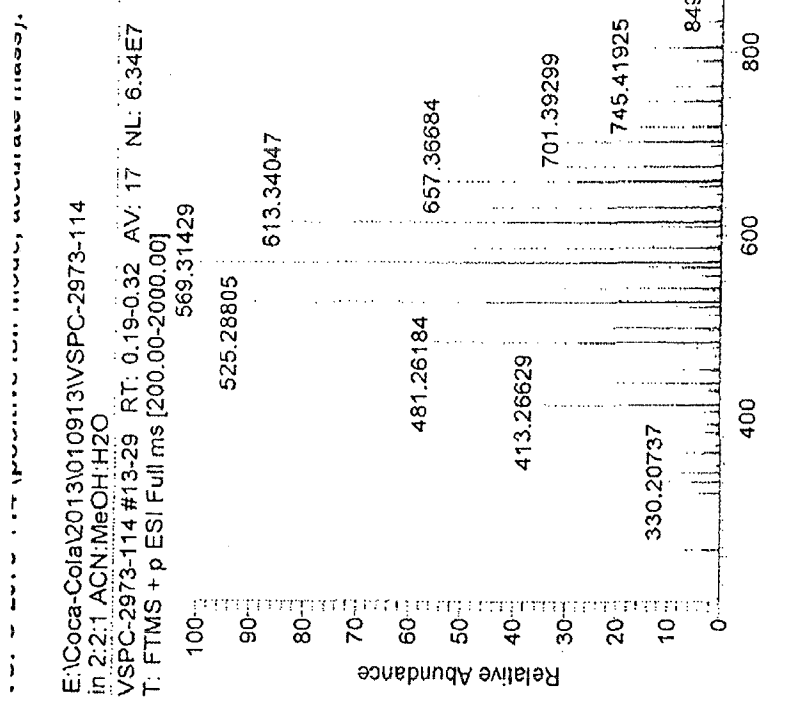


FIG. 10



The signal at *m/z* 1313.52652 is consistent with the sodium adduct of a species with molecular formula $C_{56}H_{90}O_{33}$ (+0.658 ppm). *Sample was dissolved in methanol and eluted in 2:2:1 methanol:acetonitrile:water.*

FIG. 11

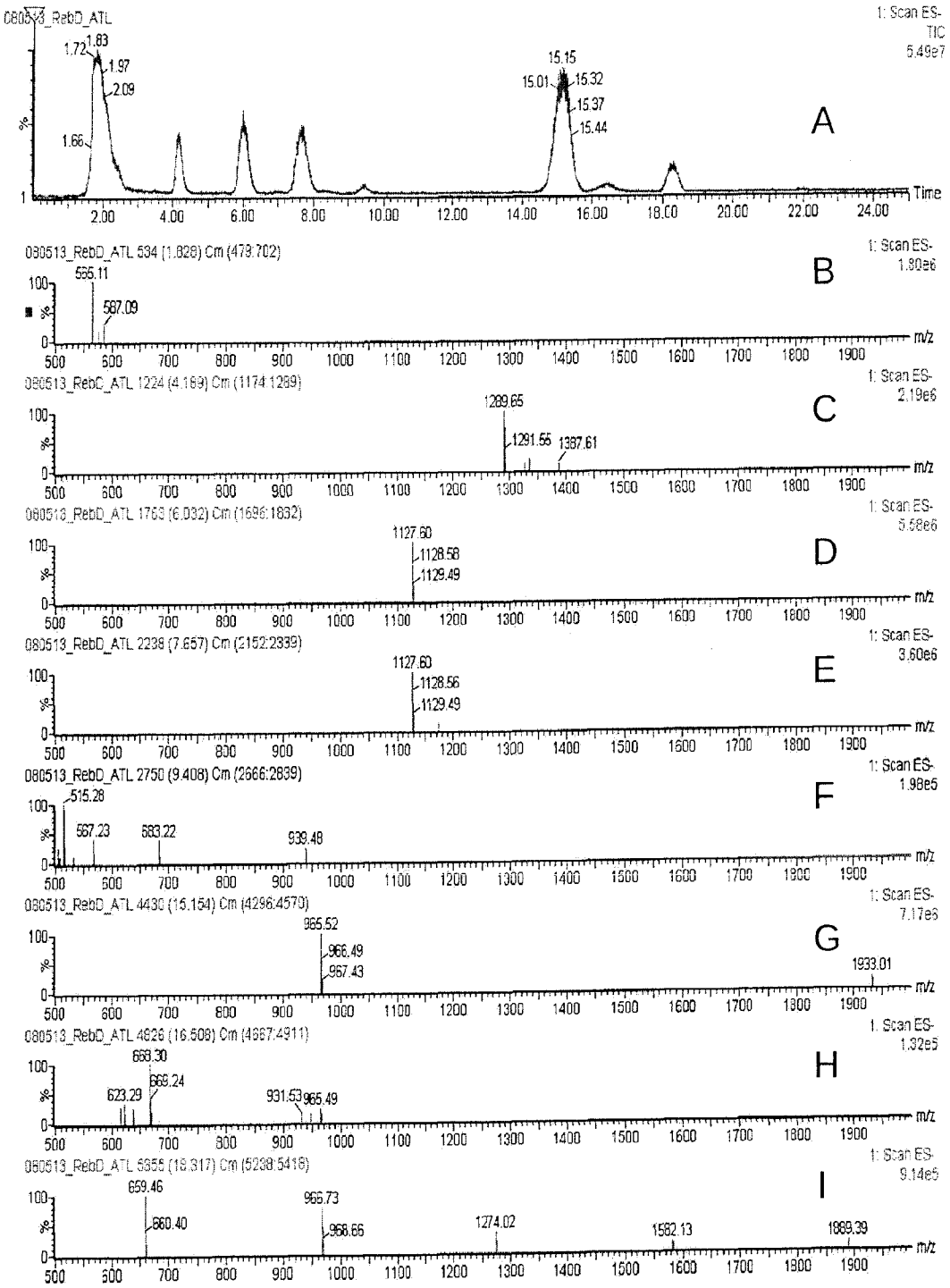

080513_RebD_ATL
1: Scan ES-
TIC
5.49e7
1.72
1.83
1.97
2.09
1.66
15.15
15.01
15.32
15.37
15.44
A
Time
B
C
D
E
F
G
H
I
m/z

FIG. 12

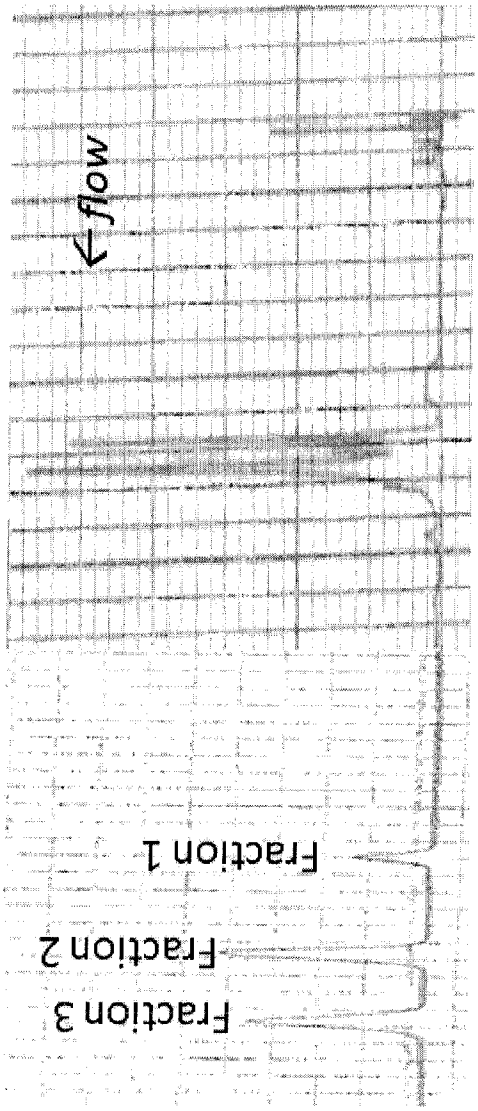


FIG. 13

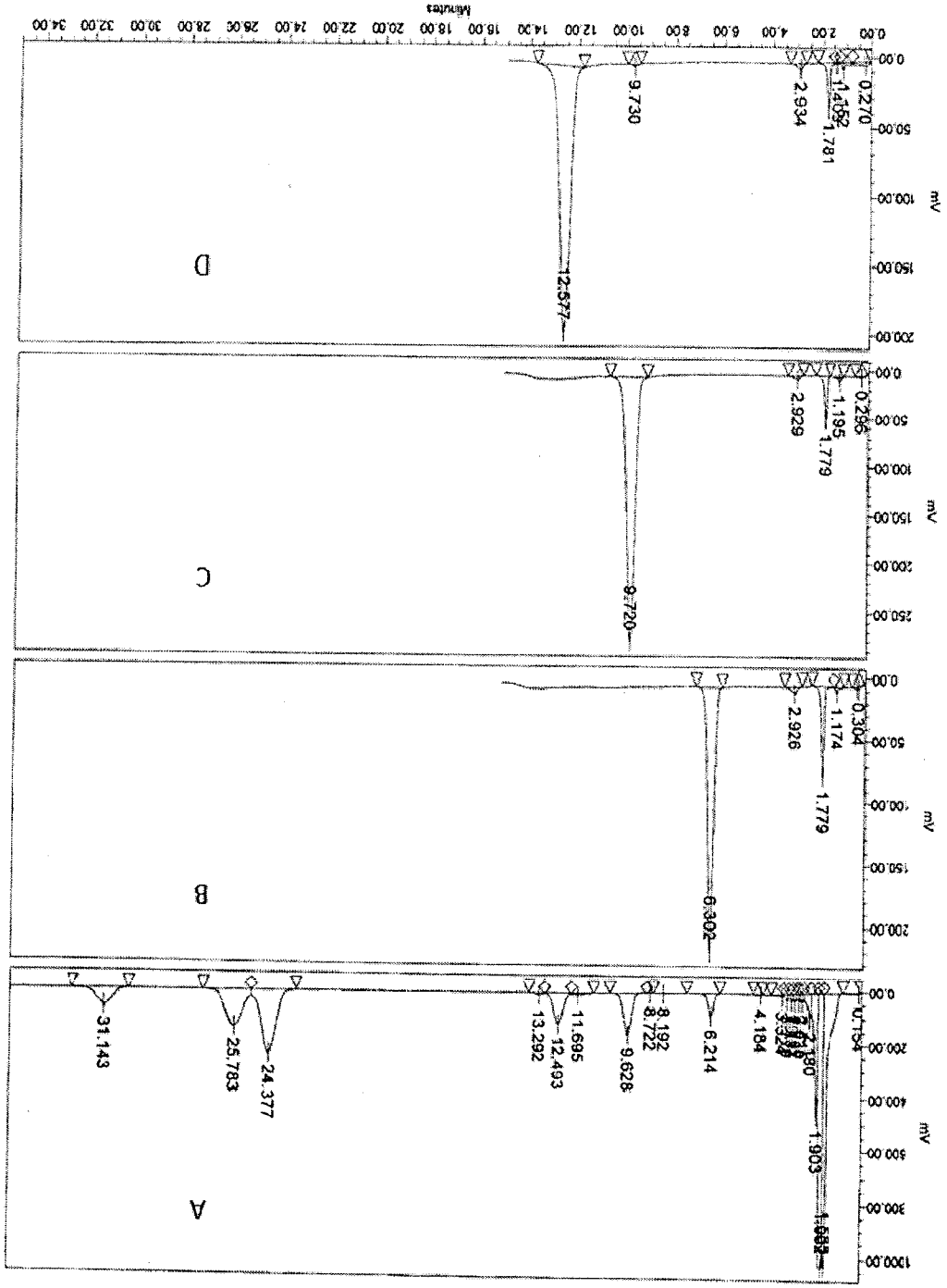

Minutes
0.00 2.00 4.00 6.00 8.00 10.00 12.00 14.00 16.00 18.00 20.00 22.00 24.00 26.00 28.00 30.00 32.00 34.00
D
0.270
9.730
2.934
1.781
12.577
C
0.286
2.929
1.195
1.779
9.720
B
0.304
2.926
1.174
1.779
6.302
A
31.143
25.783
24.377
13.292
12.493
11.695
9.628
8.722
8.192
6.214
4.184
1.903
mV

FIG. 14

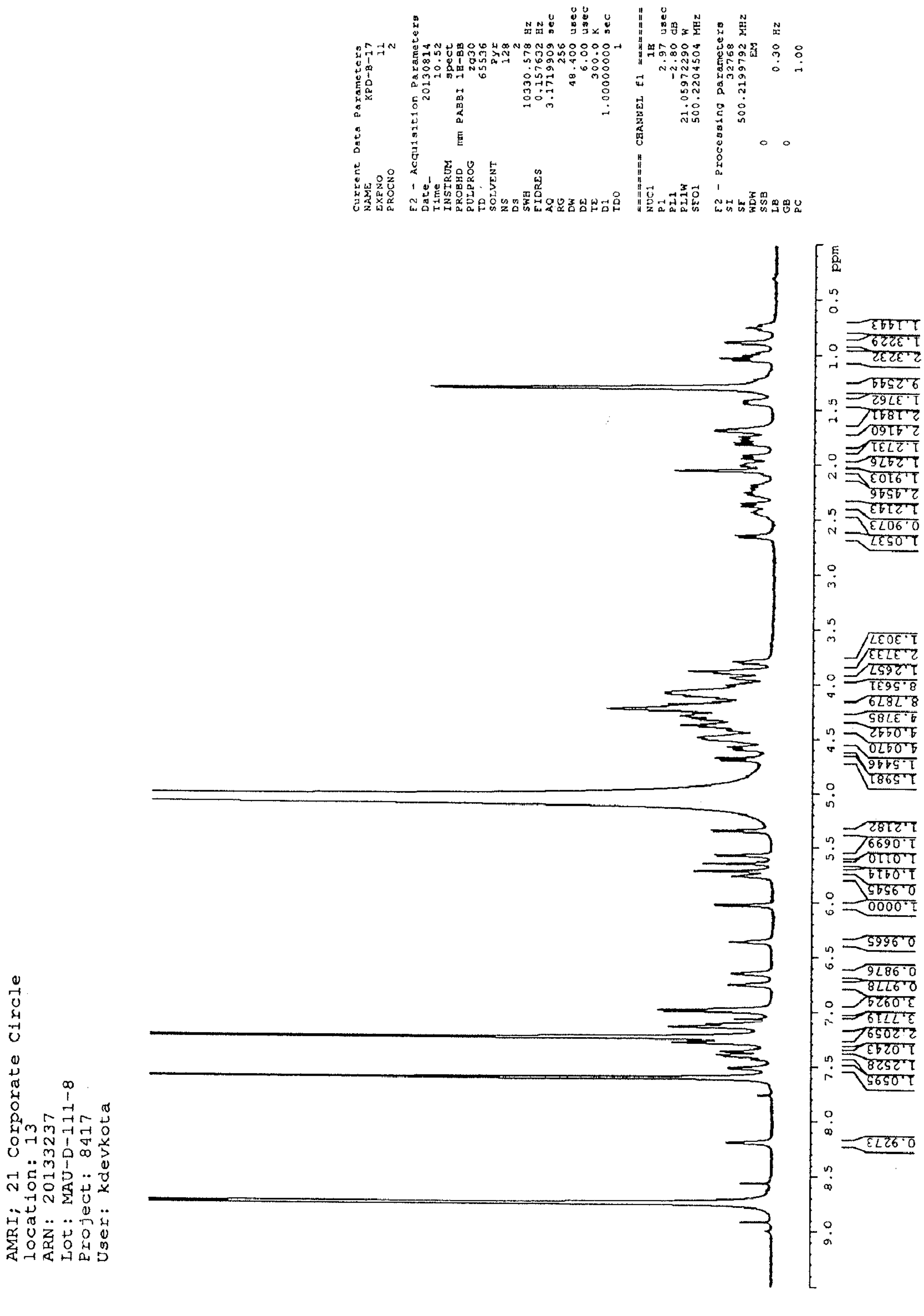

AMRI; 21 Corporate Circle
location: 13
ARN: 20133237
Lot: MAU-D-111-8
Project: 8417
User: kdevkota
ppm

FIG. 15

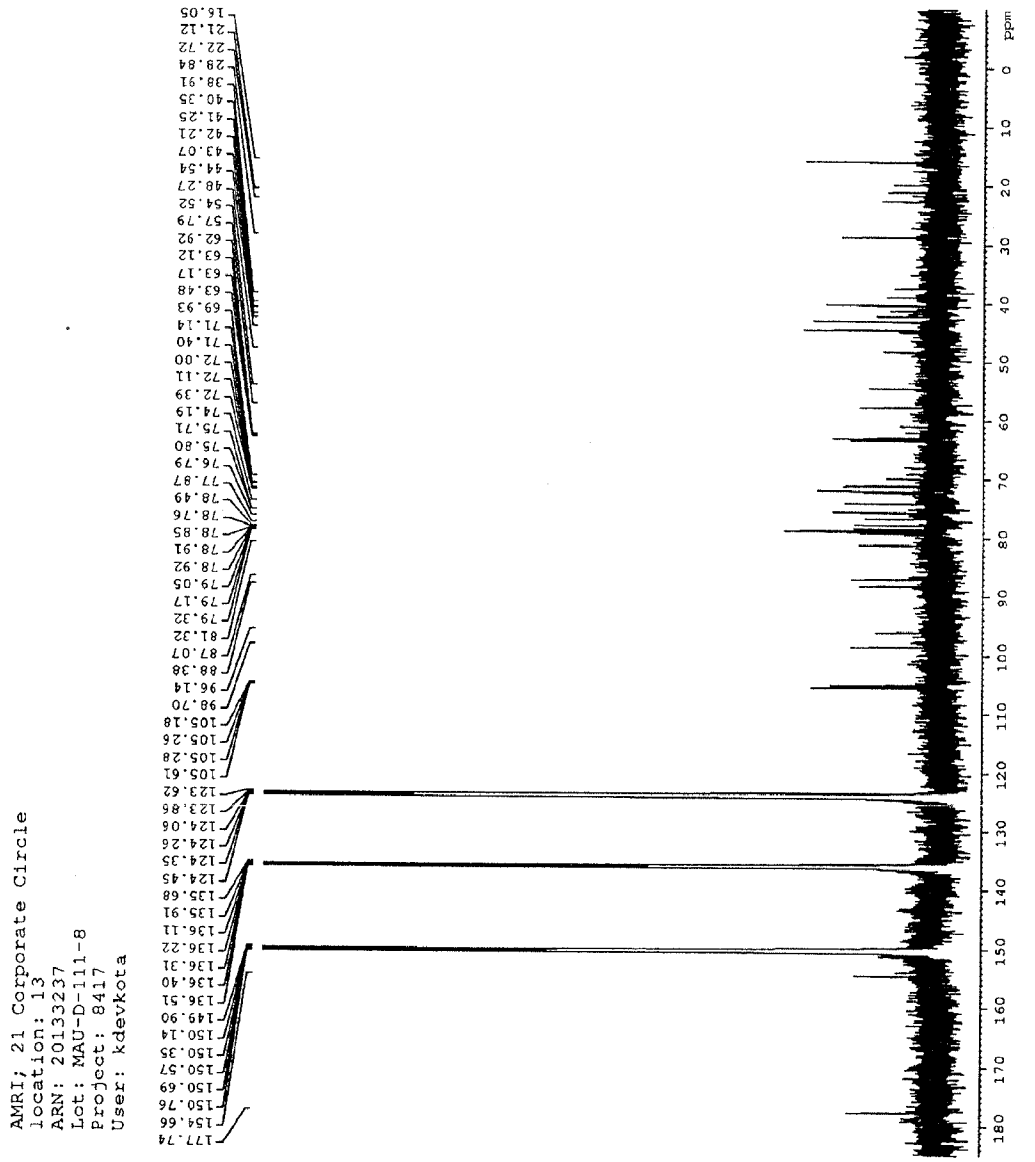

Current Data Parameters
NAME KPD-B-17
EXPNO 59
PROCNO 14
F2 - Acquisition Parameters
Date_ 20130905
Time_ 18.55
INSTRUM spect
PROBHD nm PABBI 1H-BB
PULPROG zgpg30
TD 65536
SOLVENT Pyr
NS 295992
DS 4
SWH 30030.029 Hz
FIDRES 0.458222 Hz
AQ 1.0911911 sec
RG 2048
DW 16.650 usec
DE 6.00 usec
TE 300.0 K
D1 0.50000000 sec
D11 0.03000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 13C
P1 14.50 usec
PL1 -3.00 dB
PL1W 125.29680634 W
SFO1 125.7929971 MHz
======== CHANNEL f2 ========
CPDPRG[2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -2.80 dB
PL12 25.81 dB
PL13 120.00 dB
PL2W 21.05972290 W
PL12W 0.02900366 W
PL13W 0 W
SFO2 500.2220009 MHz
F2 - Processing parameters
SI 32768
SF 125.7803247 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00
AMRI; 21 Corporate Circle
location: 13
ARN: 20133237
Lot: MAU-D-111-8
Project: 8417
User: kdevkota
ppm

FIG. 16

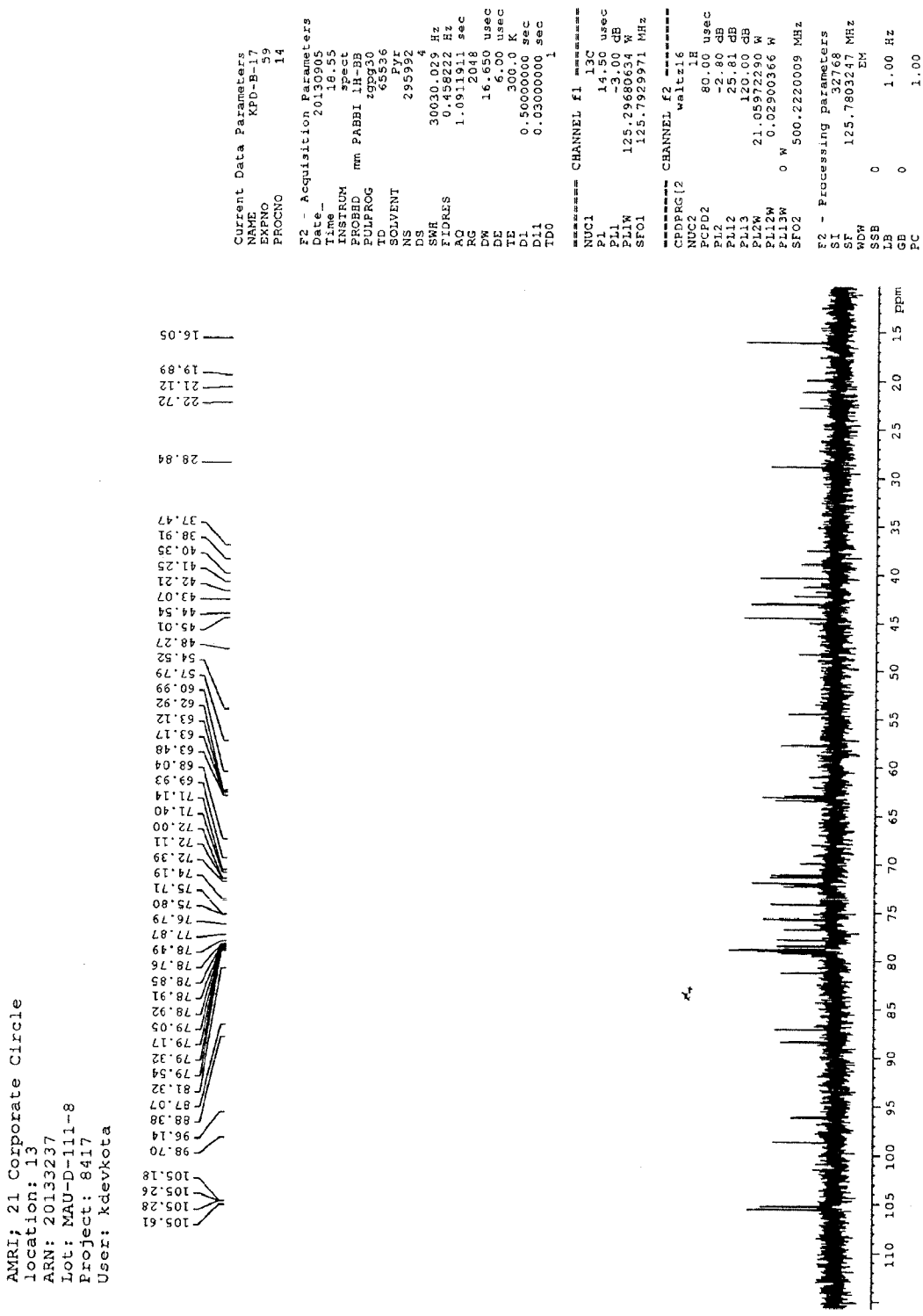

AMRI; 21 Corporate Circle
location: 13
ARN: 20133237
Lot: MAU-D-111-8
Project: 8417
User: kdevkota
Current Data Parameters
NAME KPD-B-17
EXPNO 59
PROCNO 14
F2 - Acquisition Parameters
Date_ 20130905
Time_ 18.55
INSTRUM spect
PROBHD mm PABBI 1H-BB
PULPROG zgpg30
TD 65536
SOLVENT Pyr
NS 295992
DS 4
SWH 30030.029 Hz
FIDRES 0.458222 Hz
AQ 1.09111911 sec
RG 2048
DW 16.650 usec
DE 6.00 usec
TE 300.0 K
D1 0.50000000 sec
D11 0.03000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 13C
P1 14.50 usec
PL1 -3.00 dB
PL1W 125.29680634 W
SFO1 125.7929971 MHz
======== CHANNEL f2 ========
CPDPRG[2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 -2.80 dB
PL12 25.81 dB
PL13 120.00 dB
PL2W 21.05972290 W
PL12W 0.02900366 W
PL13W 0 W
SFO2 500.2220009 MHz
F2 - Processing parameters
SI 32768
SF 125.7803247 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 1.00
16.05
19.89
21.12
22.72
28.84
37.47
38.91
40.35
41.25
42.21
43.07
44.54
45.01
48.27
54.52
57.79
60.99
62.92
63.12
63.17
63.48
68.04
69.93
71.14
71.40
72.00
72.11
72.39
74.19
75.71
75.80
76.79
77.87
78.49
78.76
78.85
78.91
78.92
79.05
79.17
79.32
79.54
81.32
87.07
88.38
96.14
98.70
105.18
105.26
105.28
105.61
ppm

FIG. 17

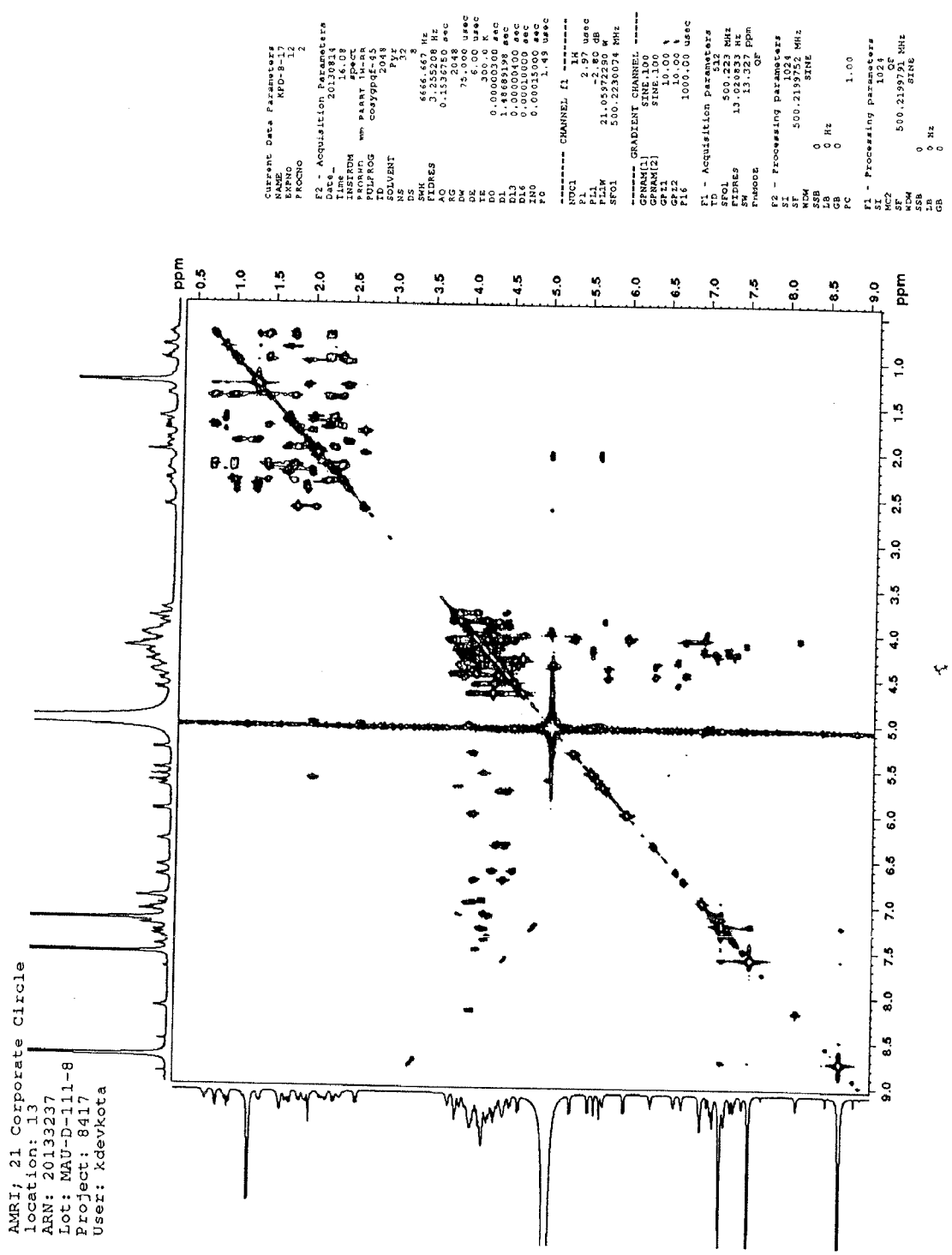

AMRI; 21 Corporate Circle
location: 13
ARN: 20133237
Lot: MAU-D-111-8
Project: 8417
User: kdevkota
ppm
ppm

FIG. 18

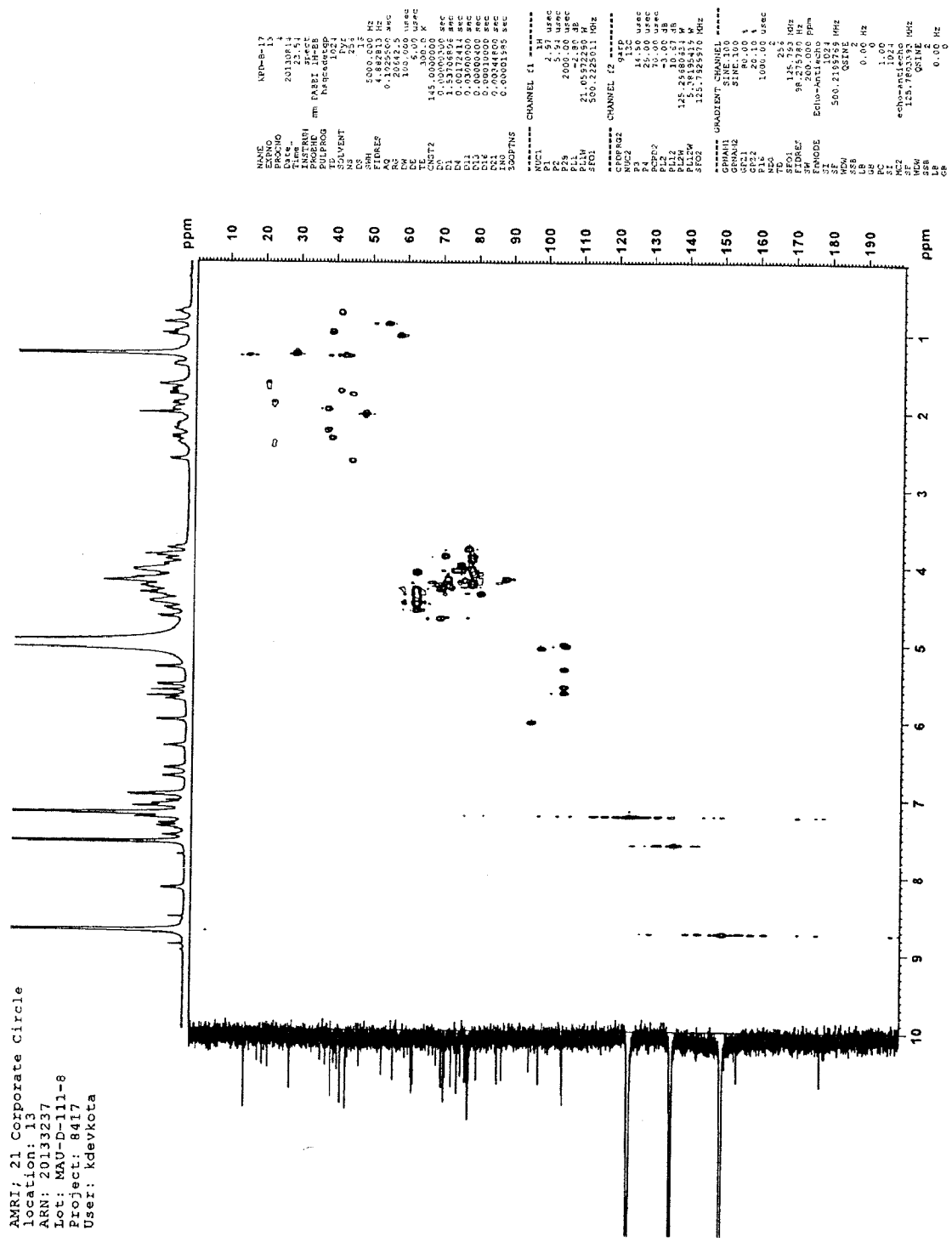

AMRI; 21 Corporate Circle
location: 13
ARN: 20133237
Iot: MAU-D-111-8
Project: 8417
User: kdevkota
ppm
ppm

FIG. 19

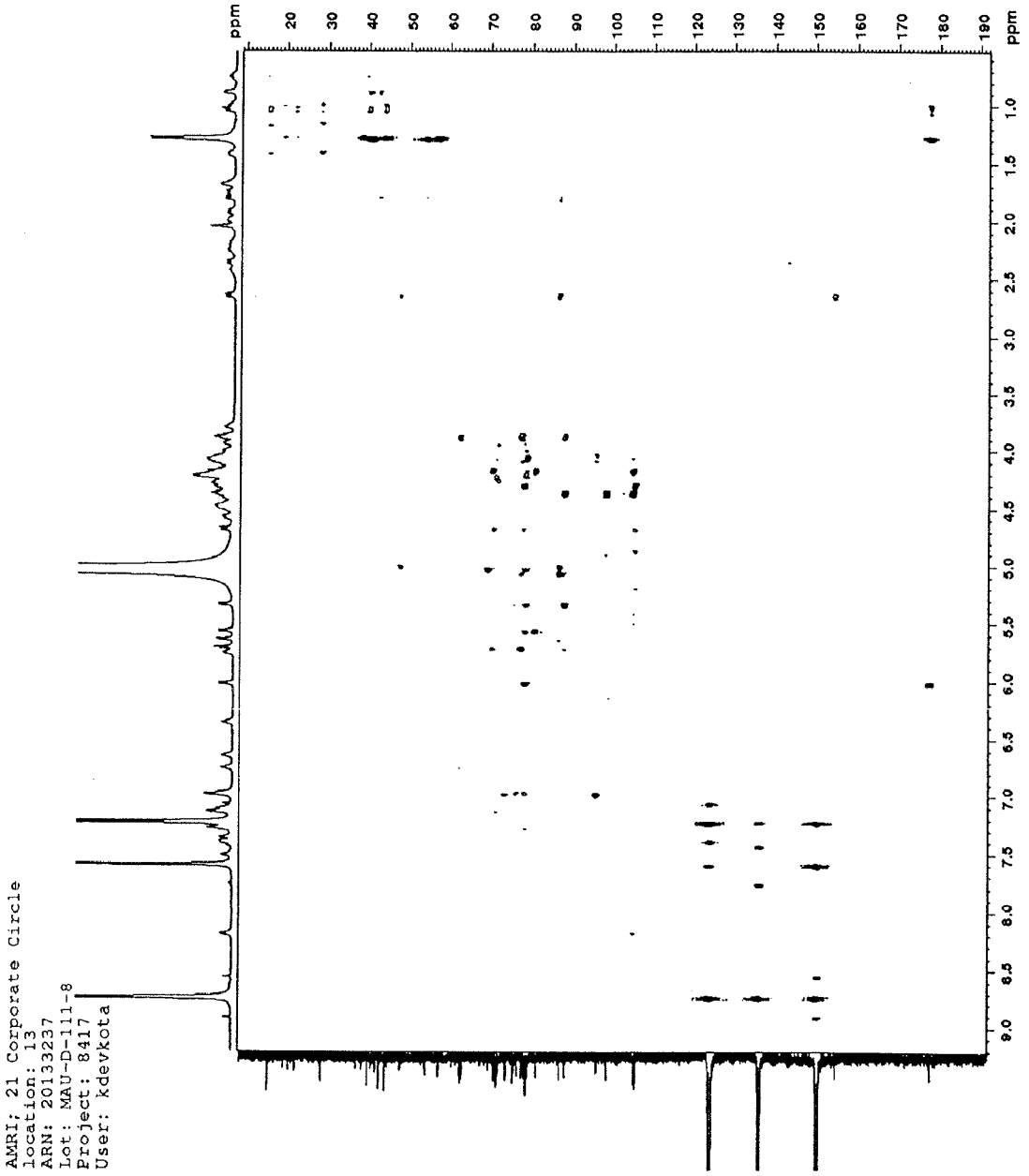

AMRI; 21 Corporate Circle
location: 13
ARN: 20133237
Lot: MAU-D-111-8
Project: 8417
User: kdevkota
ppm

FIG. 20

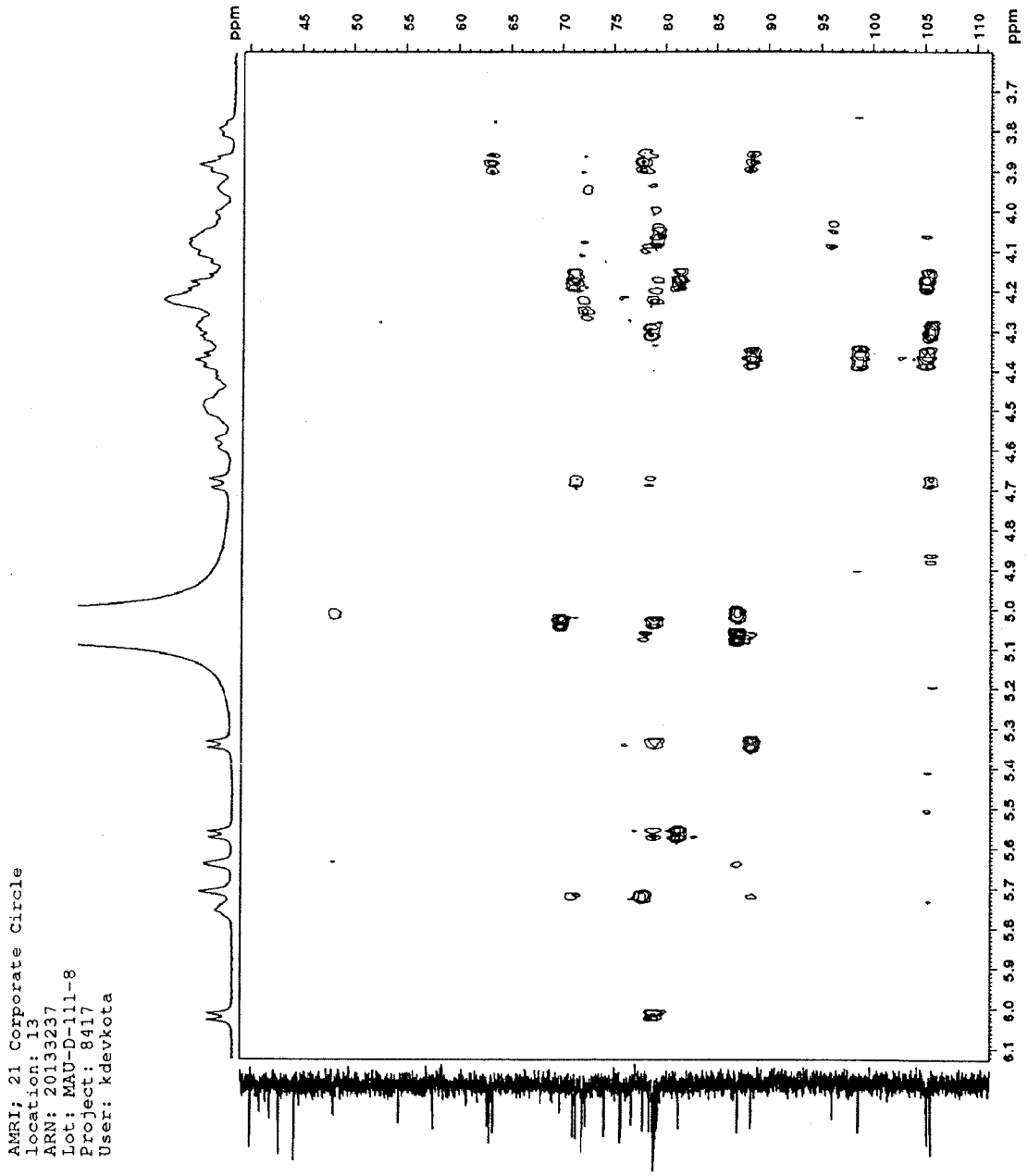

AMRI; 21 Corporate Circle
location: 13
ARN: 20133237
Lot: MAU-D-111-8
Project: 8417
User: kdevkota
ppm
ppm

FIG. 21

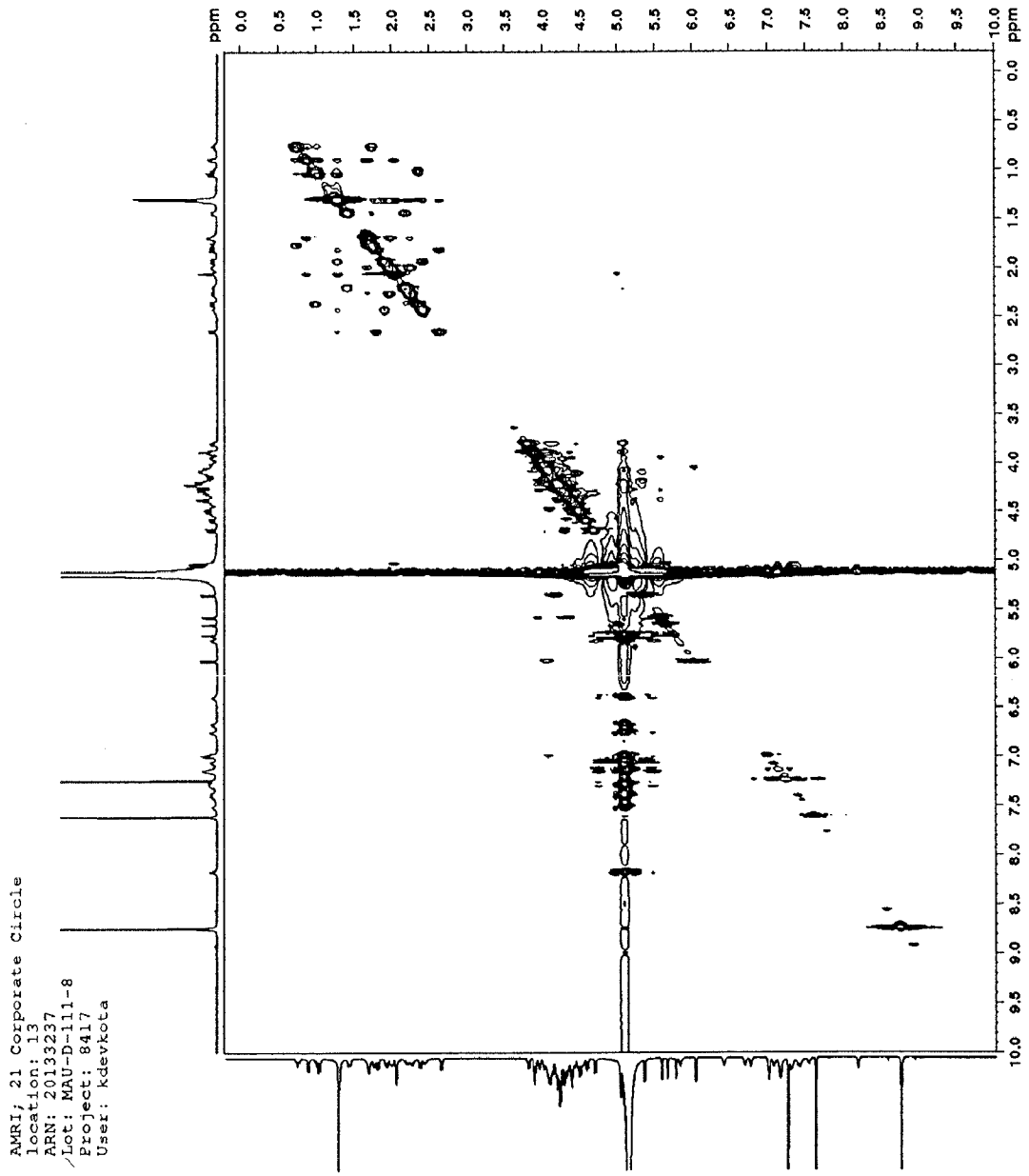

AMRI; 21 Corporate Circle
location: 13
ARN: 20133237
Lot: MAU-D-111-8
Project: 8417
User: kdevkota
ppm

FIG. 22

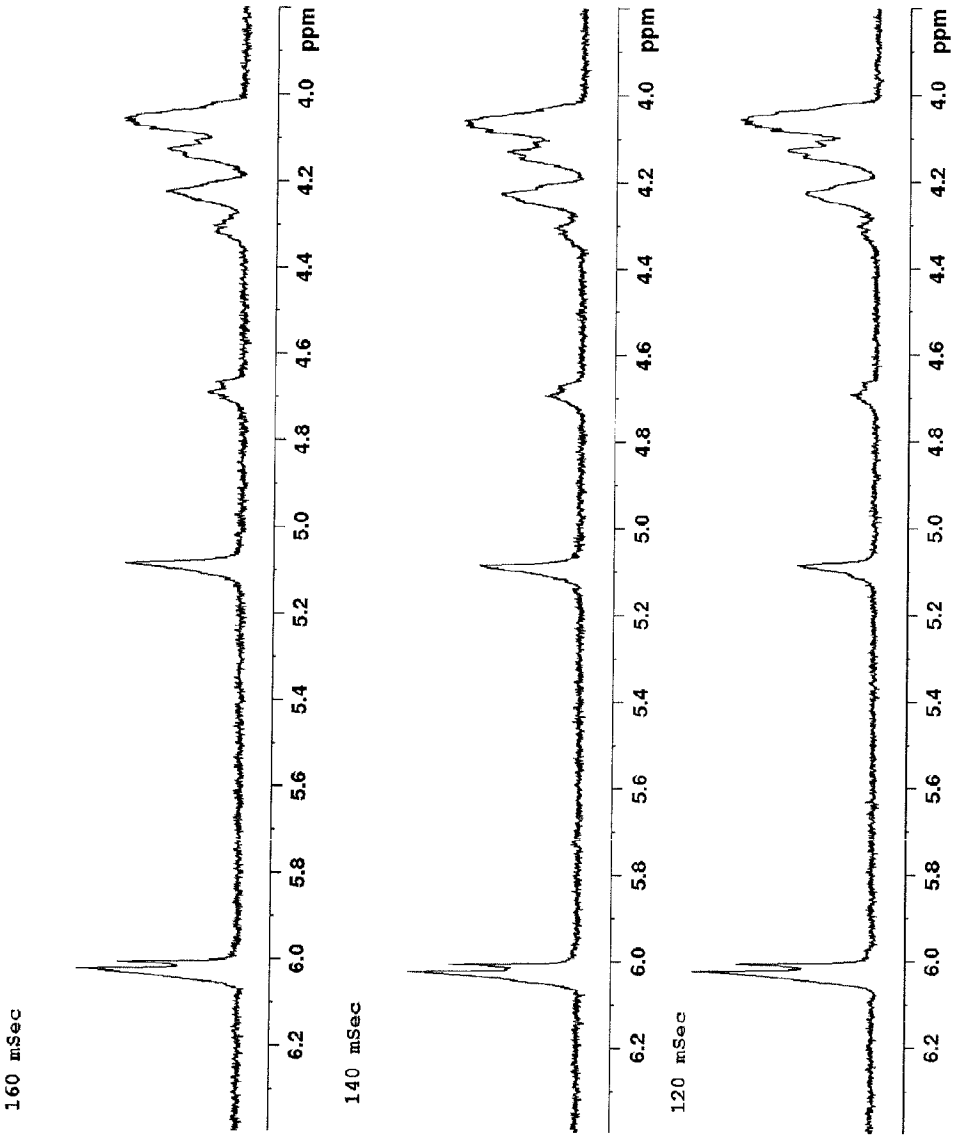


FIG. 23

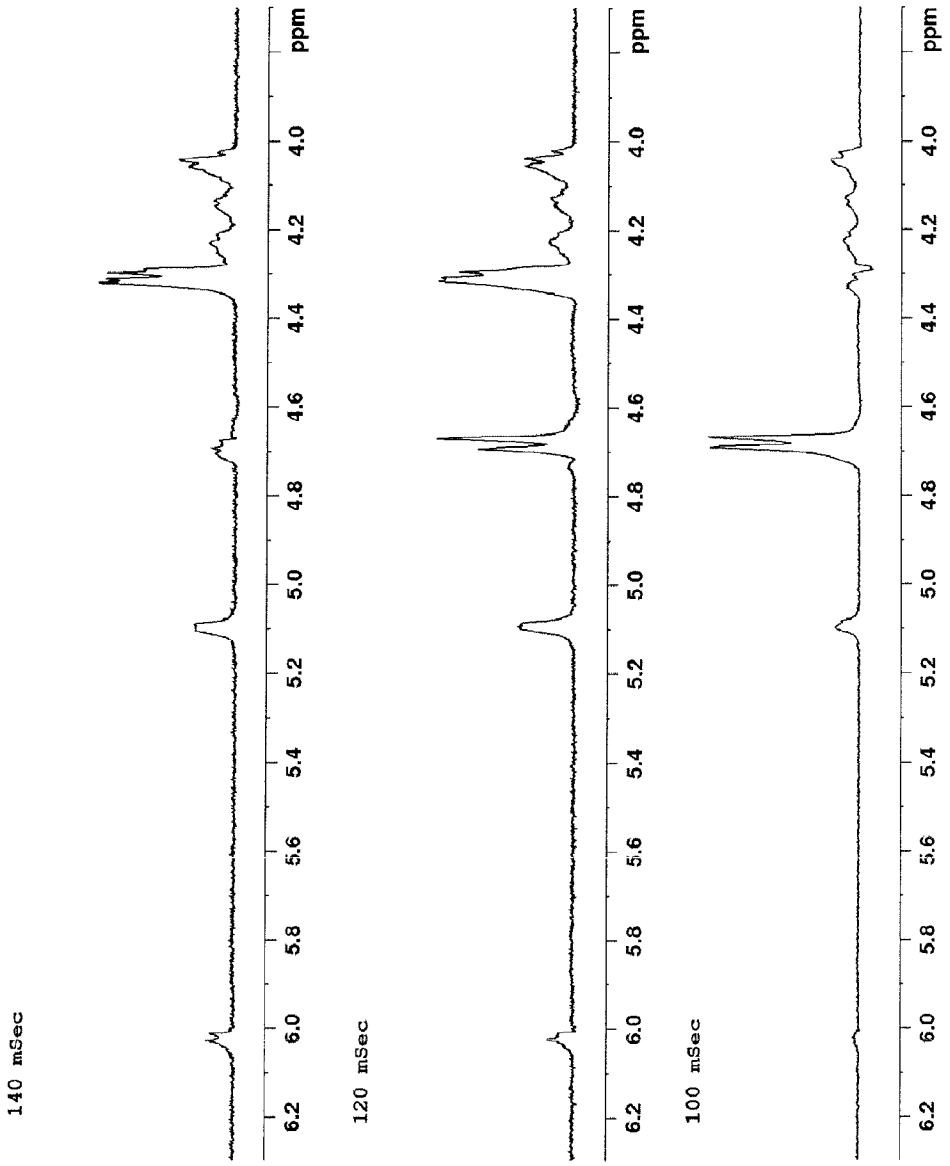


FIG. 24

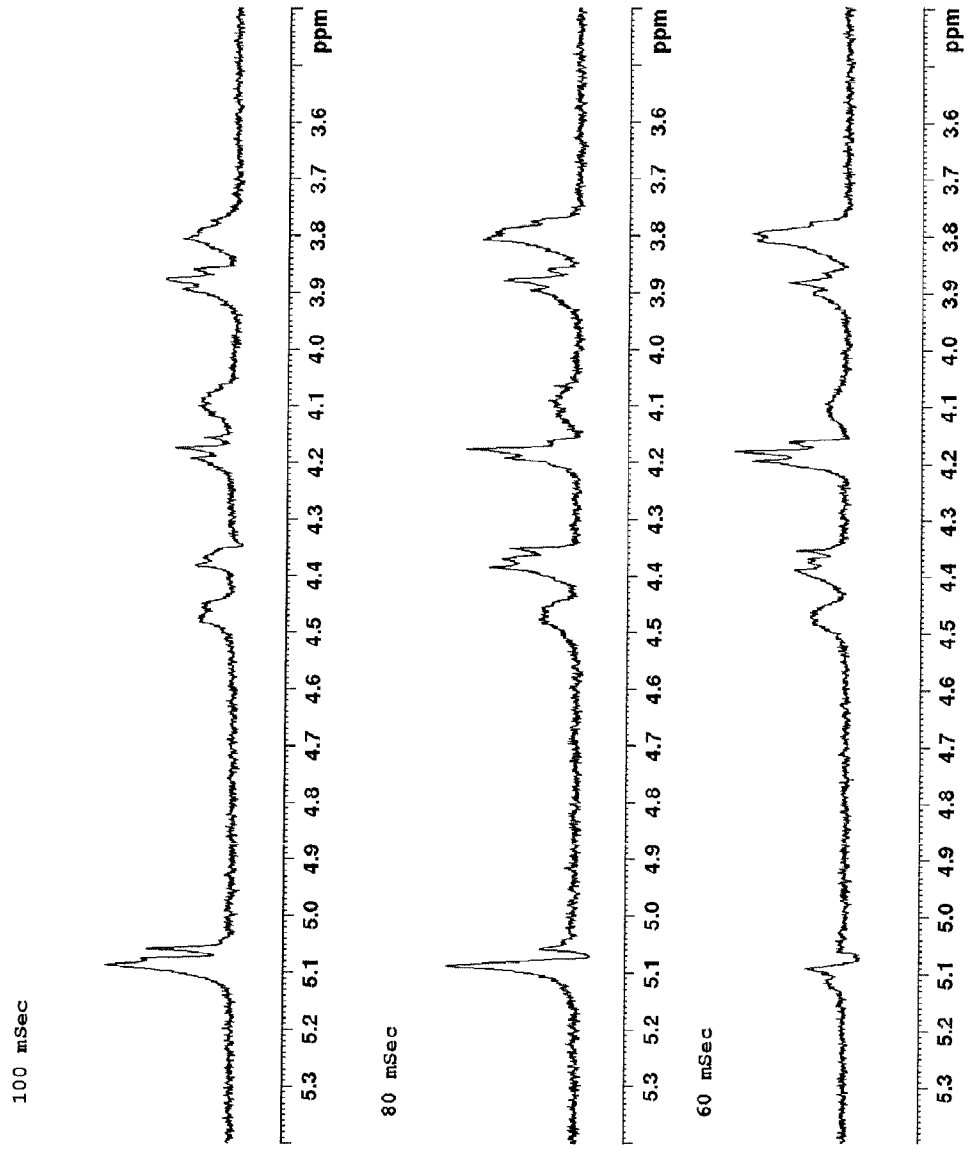

100 mSec
80 mSec
60 mSec
5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6
ppm

FIG. 25

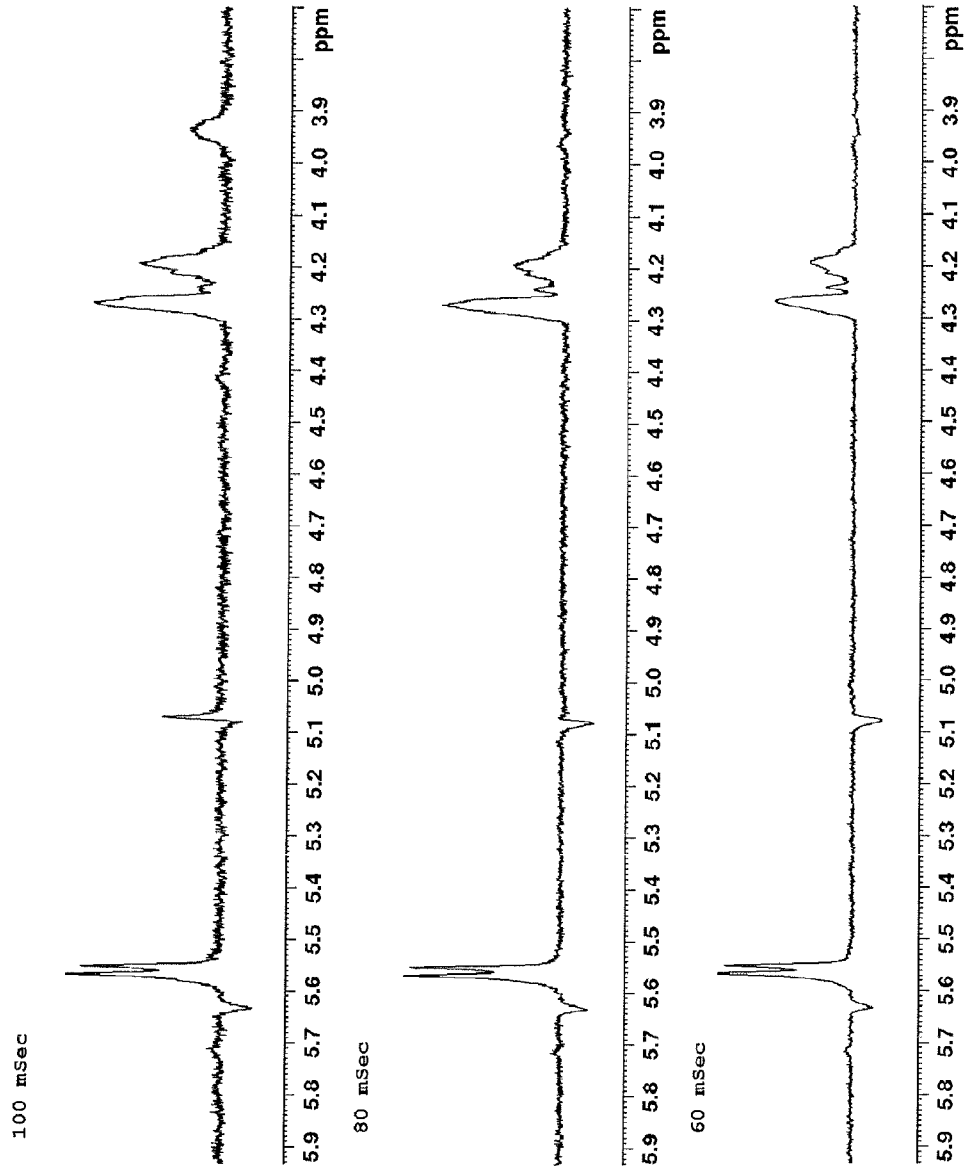

100 mSec
80 mSec
60 mSec
5.9 5.8 5.7 5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 ppm

FIG. 26

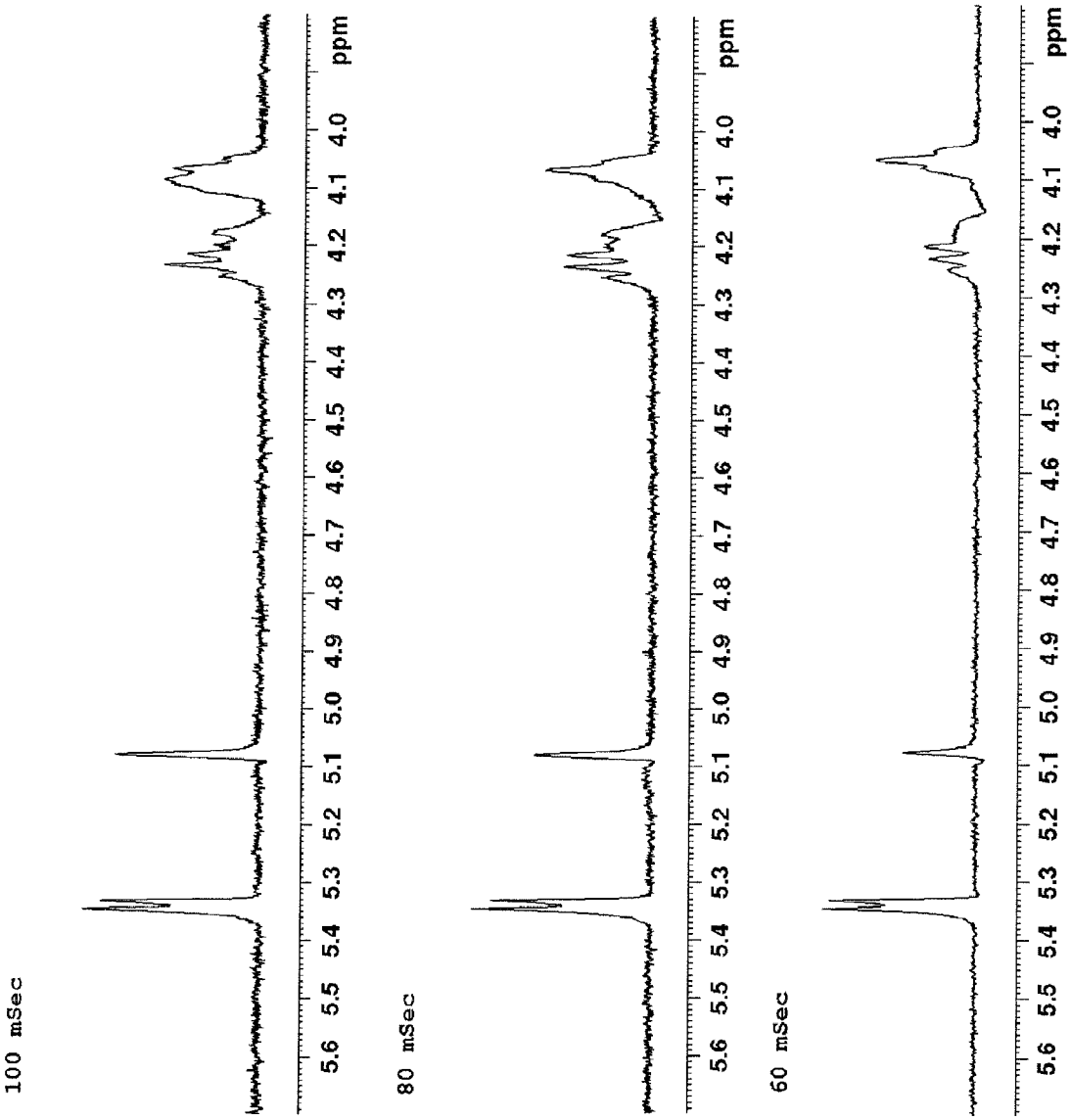

100 mSec
80 mSec
60 mSec
5.6 5.5 5.4 5.3 5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 ppm

FIG. 27

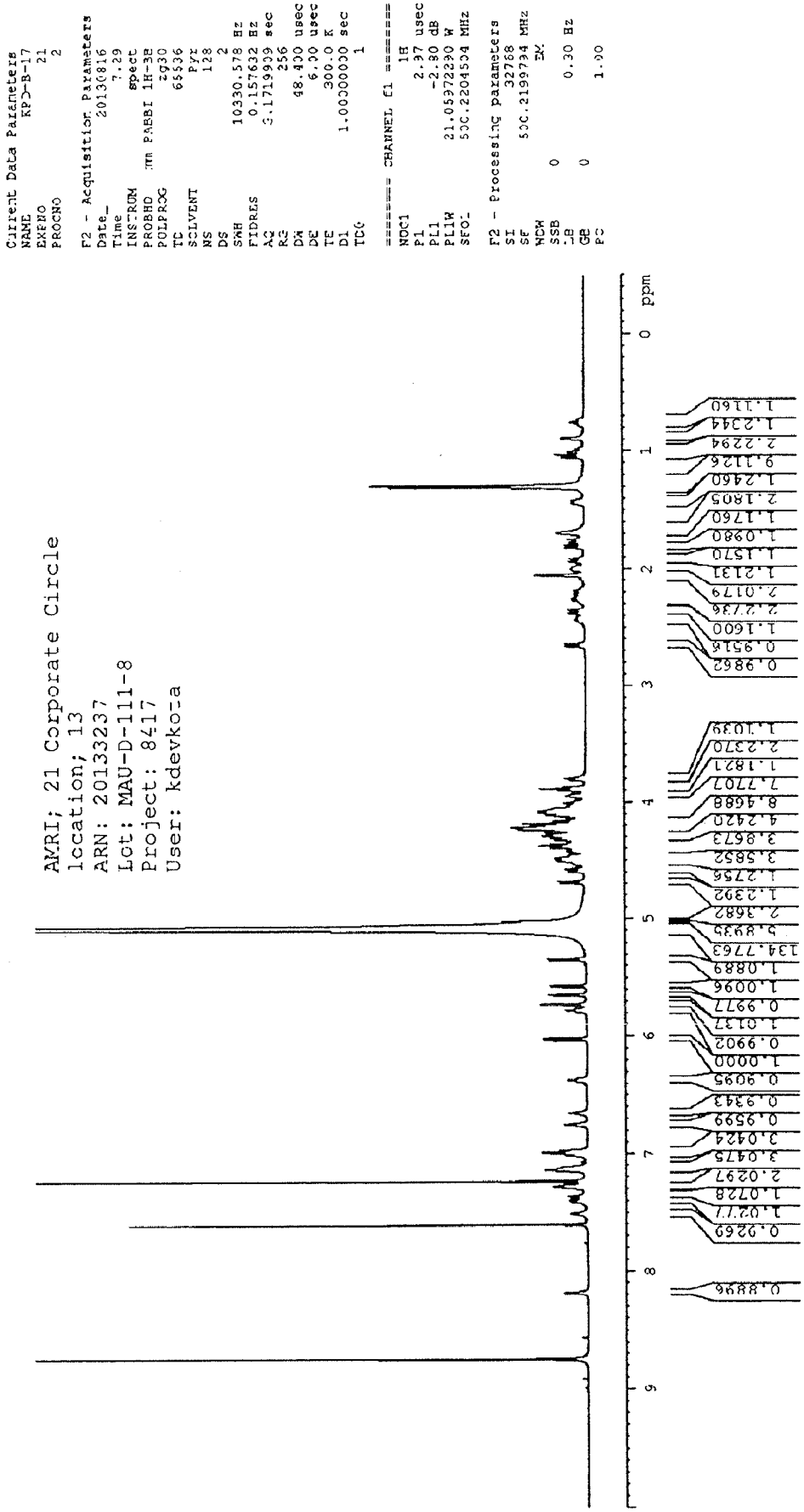
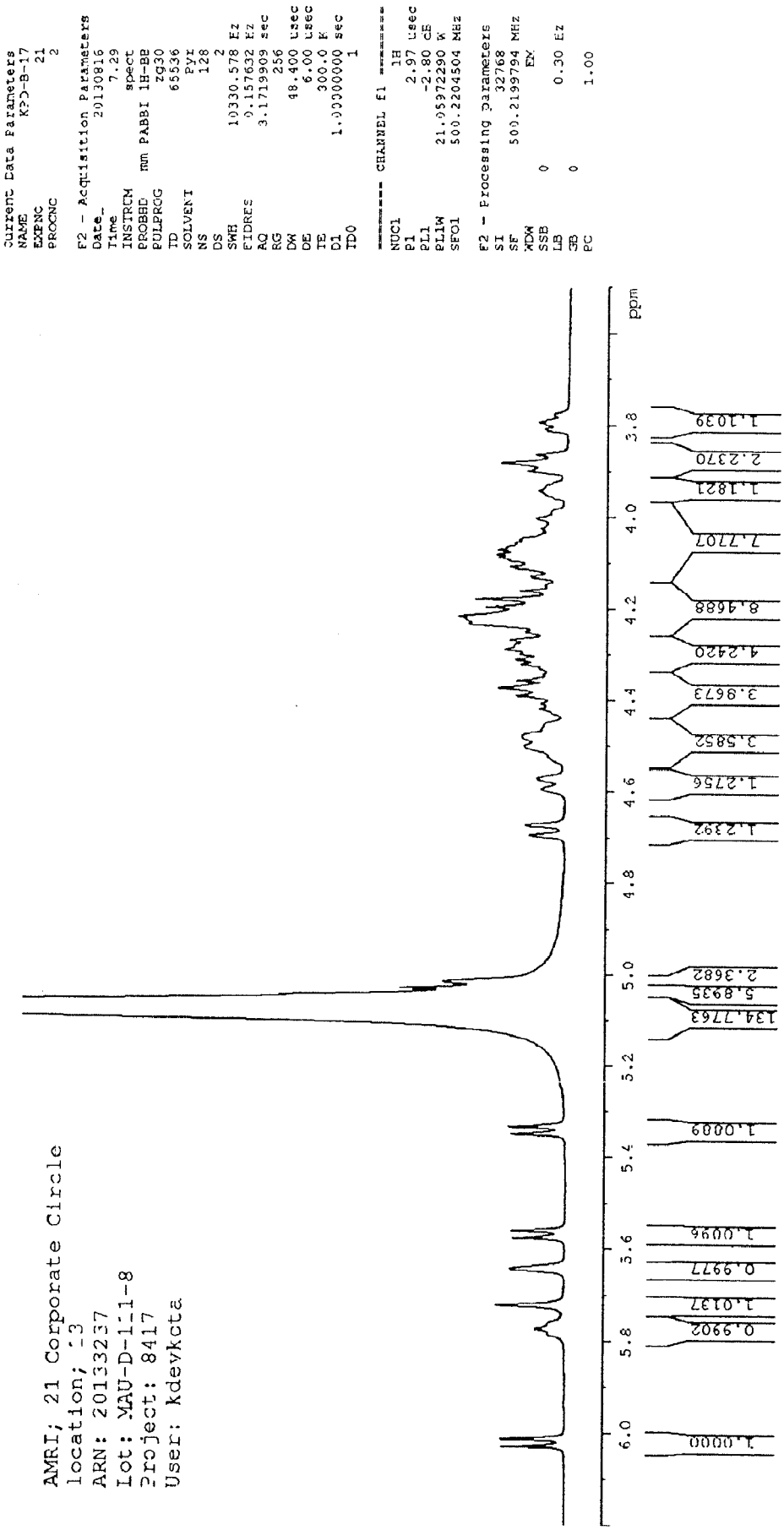

AMRI; 21 Corporate Circle
location; 13
ARN: 20133237
Lot: MAU-D-111-8
Project: 8417
User: kdevkota
Current Data Parameters
NAME KPJ-B-17
EXPNO 21
PROCNO 2
F2 - Acquisition Parameters
Date_ 20130816
Time_ 7.29
INSTRUM spect
PROBHD 5mm PABBI 1H-3B
PULPROG zg30
TD 65536
SOLVENT Pyr
NS 128
DS 2
SWH 10330.578 Hz
FIDRES 0.157632 Hz
AQ 3.1719999 sec
RG 256
DW 48.400 usec
DE 6.90 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 2.97 usec
PL1 -2.80 dB
PL1W 21.05972290 W
SFO1 500.2204594 MHz
F2 - Processing parameters
SI 32768
SF 500.2199794 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
0.8996
0.9269
1.0277
1.0728
2.0297
3.0475
3.0424
0.9599
0.9343
0.9095
1.0000
0.9902
1.0137
0.9977
1.0096
1.0889
134.7763
5.8935
2.3682
1.2392
1.2756
3.5852
3.8673
4.2420
8.4688
7.7707
1.1871
2.2370
1.1039
0.9862
0.9516
1.1600
2.2736
2.0179
1.2131
1.1570
1.0980
1.1760
2.1805
1.2460
9.1126
2.2294
1.2344
1.1160
9 8 7 6 5 4 3 2 1 0 ppm

FIG. 28


Current Data Parameters
NAME K29-B-17
EXPNO 21
PROCNO 2
F2 - Acquisition Parameters
Date_ 20130816
Time 7.29
INSTRUM spect
PROBHD mm PABBI 1H-BB
PULPROG zg30
TD 65536
SOLVENT pyr
NS 128
DS 2
SWH 10330.578 Hz
FIDRES 0.157632 Hz
AQ 3.1719909 sec
RG 256
DW 48.400 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 2.97 usec
PL1 -2.80 dB
PLIW 21.05972290 W
SFO1 500.2204504 MHz
F2 - Processing parameters
SI 32768
SF 500.2199794 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
AMRI; 21 Corporate Circle
location; :3
ARN: 20133237
Lot: MAU-D-1:1-8
Project: 8417
User: kdevkcta
ppm
1.0000
0.9902
1.0137
0.9977
1.0096
1.0009
134.7763
5.8935
2.3682
1.2392
1.2756
3.5852
3.8673
4.2420
8.4688
7.7707
1.1821
2.2370
1.1039

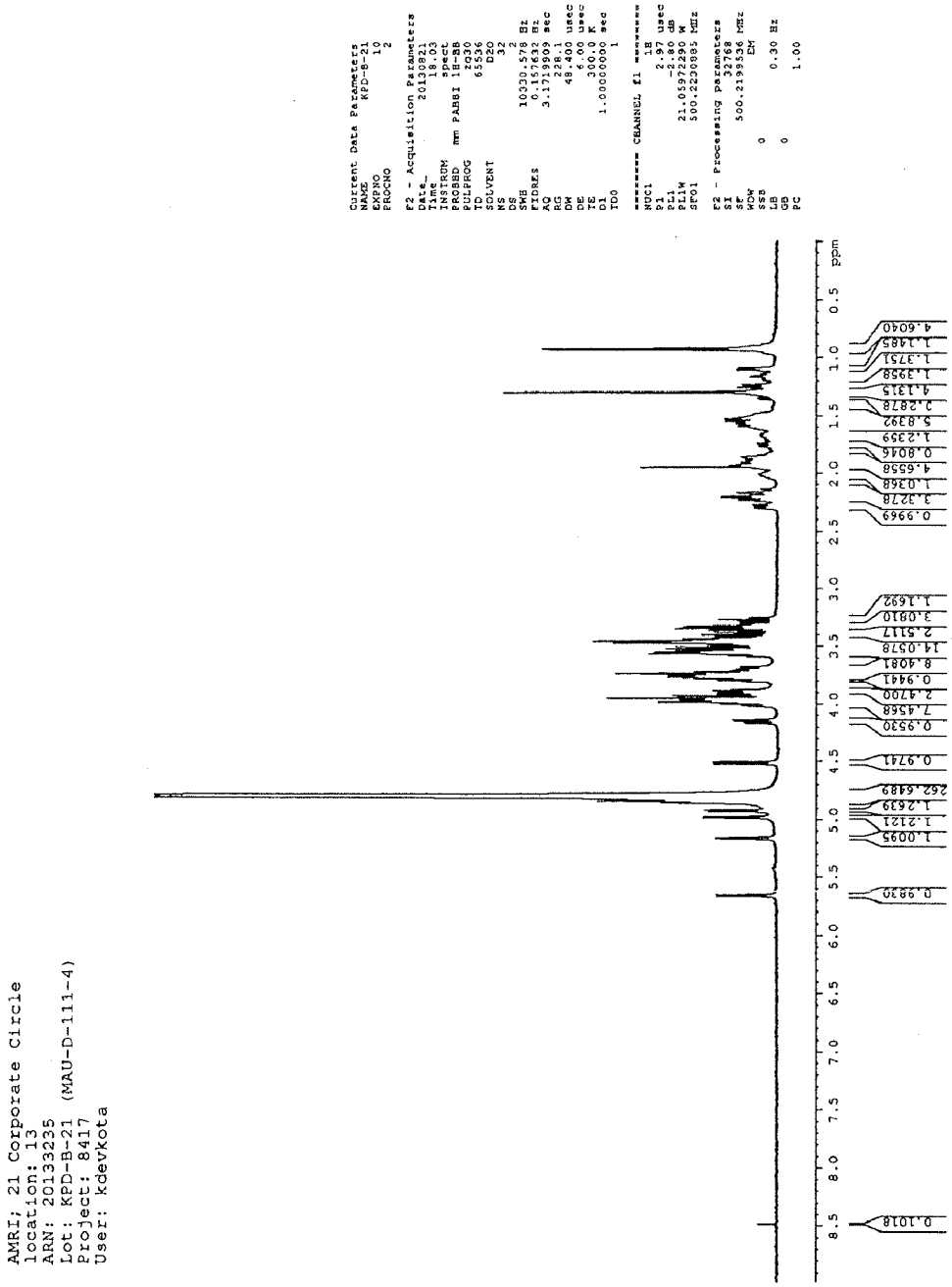

AMRI; 21 Corporate Circle
location: 13
ARN: 20133235
Lot: KPD-B-21 (MAU-D-111-4)
Project: 8417
User: kdevkota
Current Data Parameters
NAME KPD-B-21
EXPNO 10
PROCNO 2
F2 - Acquisition Parameters
Date_ 20130821
Time 18.03
INSTRUM spect
PROBHD mm PABBI 1H-BB
PULPROG zg30
TD 65536
SOLVENT D2O
NS 32
DS 2
SWH 10330.578 Hz
FIDRES 0.157632 Hz
AQ 3.1719680 sec
RG 228.1
DW 48.400 usec
DE 6.00 usec
TE 300.0 K
D1 1.00000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 1H
P1 2.97 usec
PL1 -2.00 dB
PL1W 21.05972290 W
SFO1 500.2220095 MHz
F2 - Processing parameters
SI 32768
SF 500.2199536 MHz
WDW EM
SSB 0
LB 0.30 Hz
GB 0
PC 1.00
ppm
0.1018
0.9830
1.0095
1.2121
1.2639
262.6489
0.9741
0.9530
7.4568
2.4700
0.9441
8.4081
14.0578
2.5117
3.0810
1.1692
0.9969
3.3278
1.0368
4.6558
0.8046
1.2359
5.8392
0.2878
4.1315
1.3958
1.3751
1.1485
4.6040

FIG. 29

FIG. 30

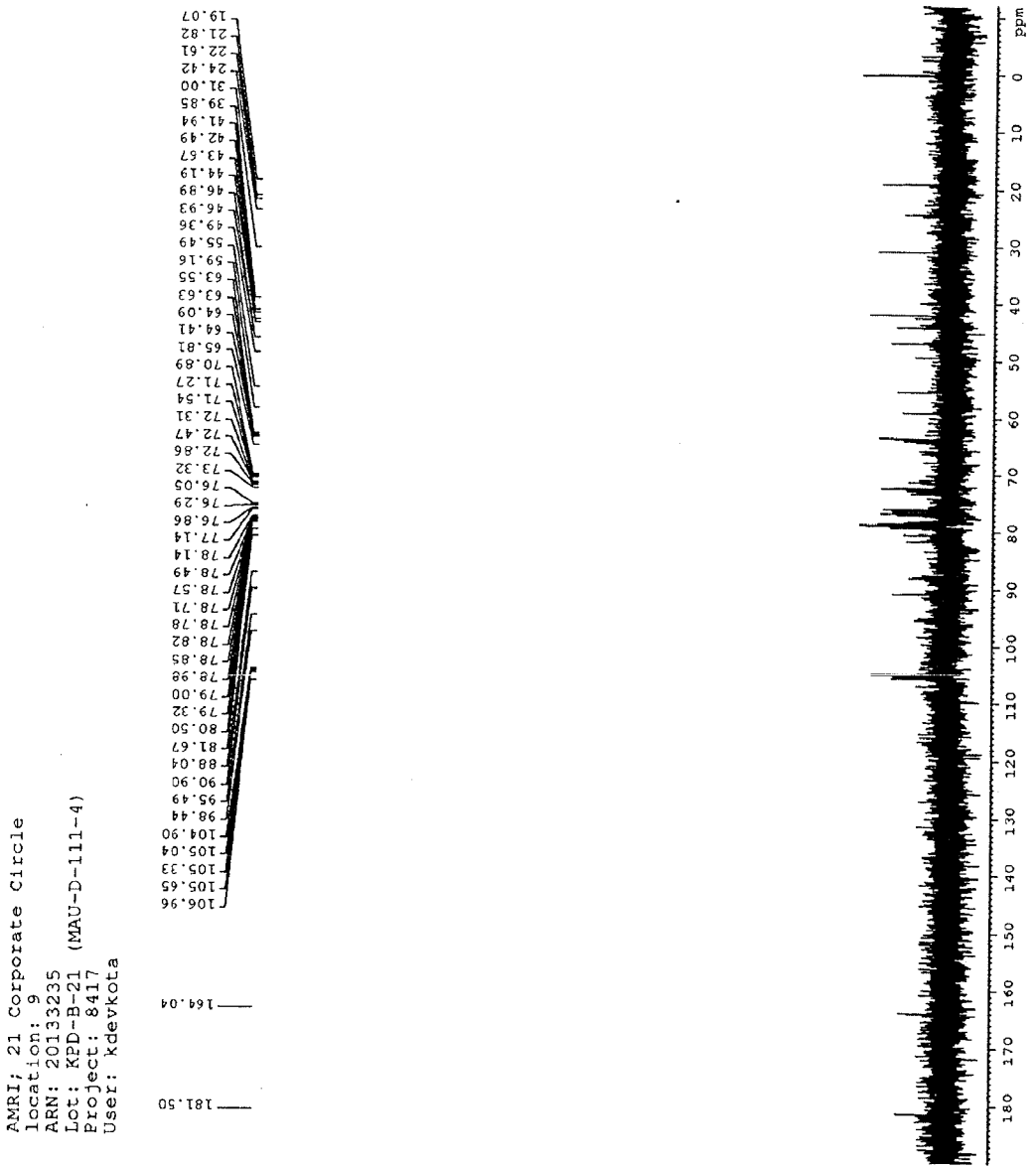

AMRI; 21 Corporate Circle
location: 9
ARN: 20133235
Lot: KPD-B-21 (MAU-D-111-4)
Project: 8417
User: kdevkota
Current Data Parameters
NAME KPD-B-21
EXPNO 12
PROCNO 2
F2 - Acquisition Parameters
Date_ 20130914
Time 17.14
INSTRUM spect
PROBHD 5 mm BBO BB-1H
PULPROG zgpg30
TD 65536
SOLVENT D2O
NS 129024
DS 4
SWH 30030.029 Hz
FIDRES 0.458222 Hz
AQ 1.0911911 sec
RG 456.1
DW 16.650 usec
DE 6.00 usec
TE 300.0 K
D1 0.50000000 sec
D11 0.03000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 13C
P1 7.75 usec
PL1 1.50 dB
PL1W 64.01805878 W
SFO1 125.7703643 MHz
======== CHANNEL f2 ========
CPDPRG[2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 0.50 dB
PL12 16.00 dB
PL13 16.50 dB
PL2W 13.43232727 W
PL12W 0.37857440 W
PL13W 0.33740479 W
SFO2 500.1320005 MHz
F2 - Processing parameters
SI 32768
SF 125.7574273 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 0.80
ppm

FIG. 31

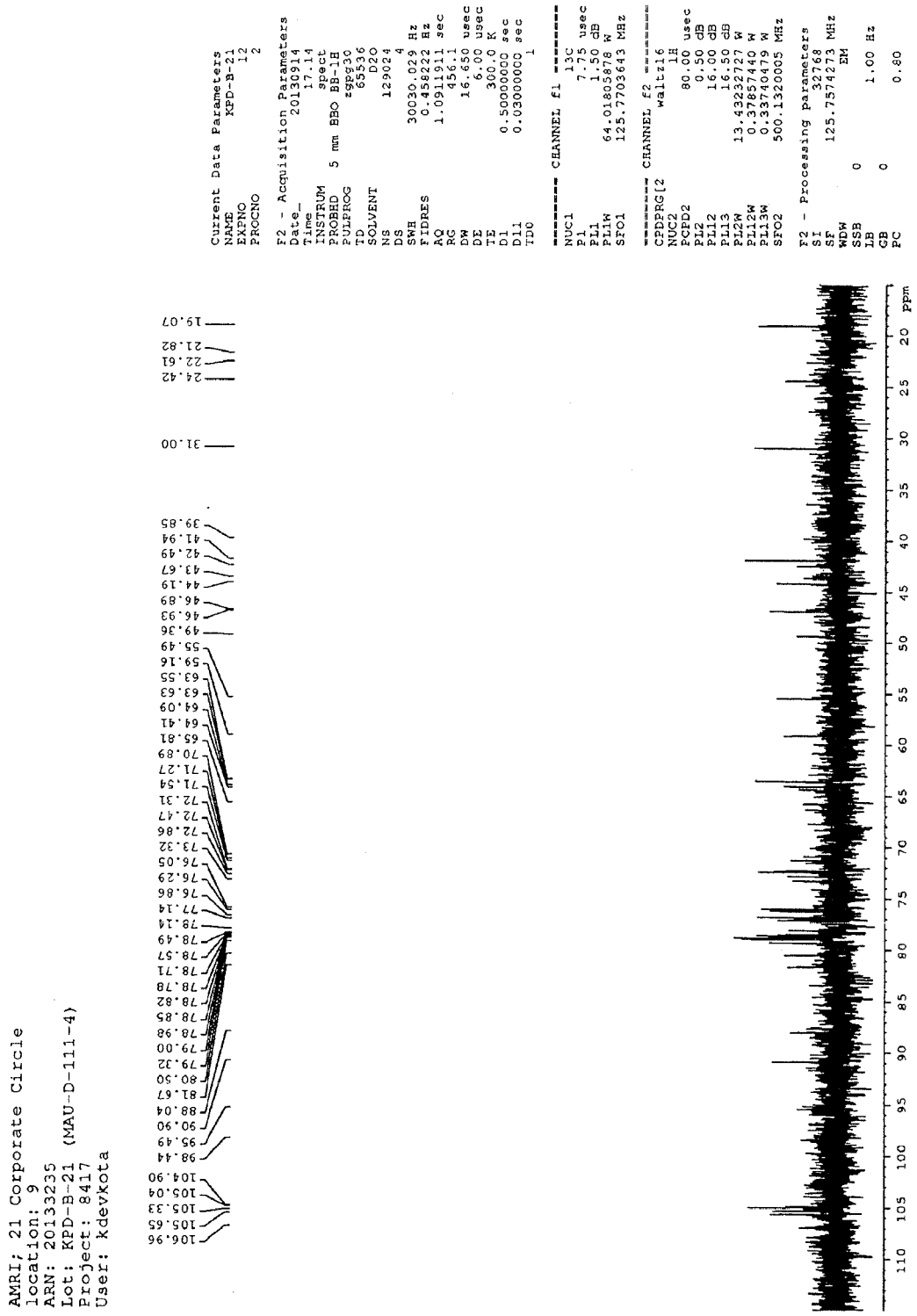

AMRI; 21 Corporate Circle
location: 9
ARN: 20133235
Lot: KPD-B-21 (MAU-D-111-4)
Project: 8417
User: kdevkota
Current Data Parameters
NAME KPD-B-21
EXPNO 12
PROCNO 2
F2 - Acquisition Parameters
Date_ 20130914
Time_ 17.14
INSTRUM spect
PROBHD 5 mm BBO BB-1H
PULPROG zgpg30
TD 65536
SOLVENT D2O
NS 129024
DS 4
SWH 30030.029 Hz
FIDRES 0.458222 Hz
AQ 1.09111911 sec
RG 456.1
DW 16.650 usec
DE 6.00 usec
TE 300.0 K
D1 0.50000000 sec
D11 0.03000000 sec
TD0 1
======== CHANNEL f1 ========
NUC1 13C
P1 7.75 usec
PL1 1.50 dB
PL1W 64.01805878 W
SFO1 125.7703643 MHz
======== CHANNEL f2 ========
CPDPRG[2 waltz16
NUC2 1H
PCPD2 80.00 usec
PL2 0.50 dB
PL12 16.00 dB
PL13 16.50 dB
PL2W 13.43232727 W
PL12W 0.37857440 W
PL13W 0.33740479 W
SFO2 500.1320005 MHz
F2 - Processing parameters
SI 32768
SF 125.7574273 MHz
WDW EM
SSB 0
LB 1.00 Hz
GB 0
PC 0.80
19.07
21.82
22.61
24.42
31.00
39.85
41.94
42.49
43.67
44.19
46.89
46.93
49.36
55.49
59.16
63.55
63.63
64.09
64.41
65.81
70.89
71.27
71.54
72.31
72.47
72.86
73.32
76.05
76.29
76.86
77.14
78.14
78.49
78.57
78.71
78.78
78.82
78.85
78.98
79.00
79.32
80.50
81.67
88.04
90.90
95.49
98.44
104.90
105.04
105.33
105.65
106.96
ppm

FIG. 32

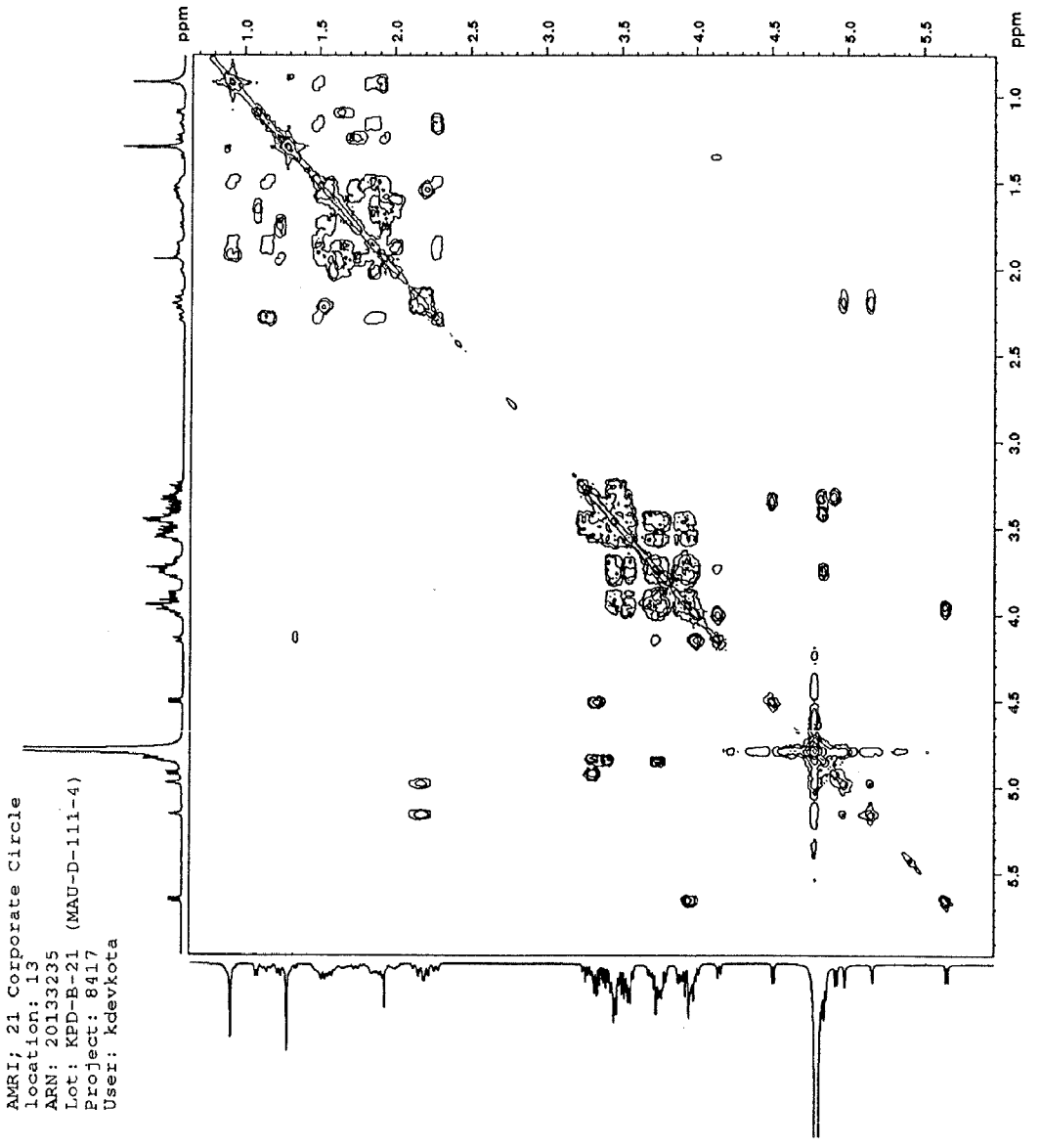

AMRI; 21 Corporate Circle
location: 13
ARN: 20133235
Lot: KPD-B-21 (MAU-D-111-4)
Project: 8417
User: kdevkota
ppm

FIG. 33

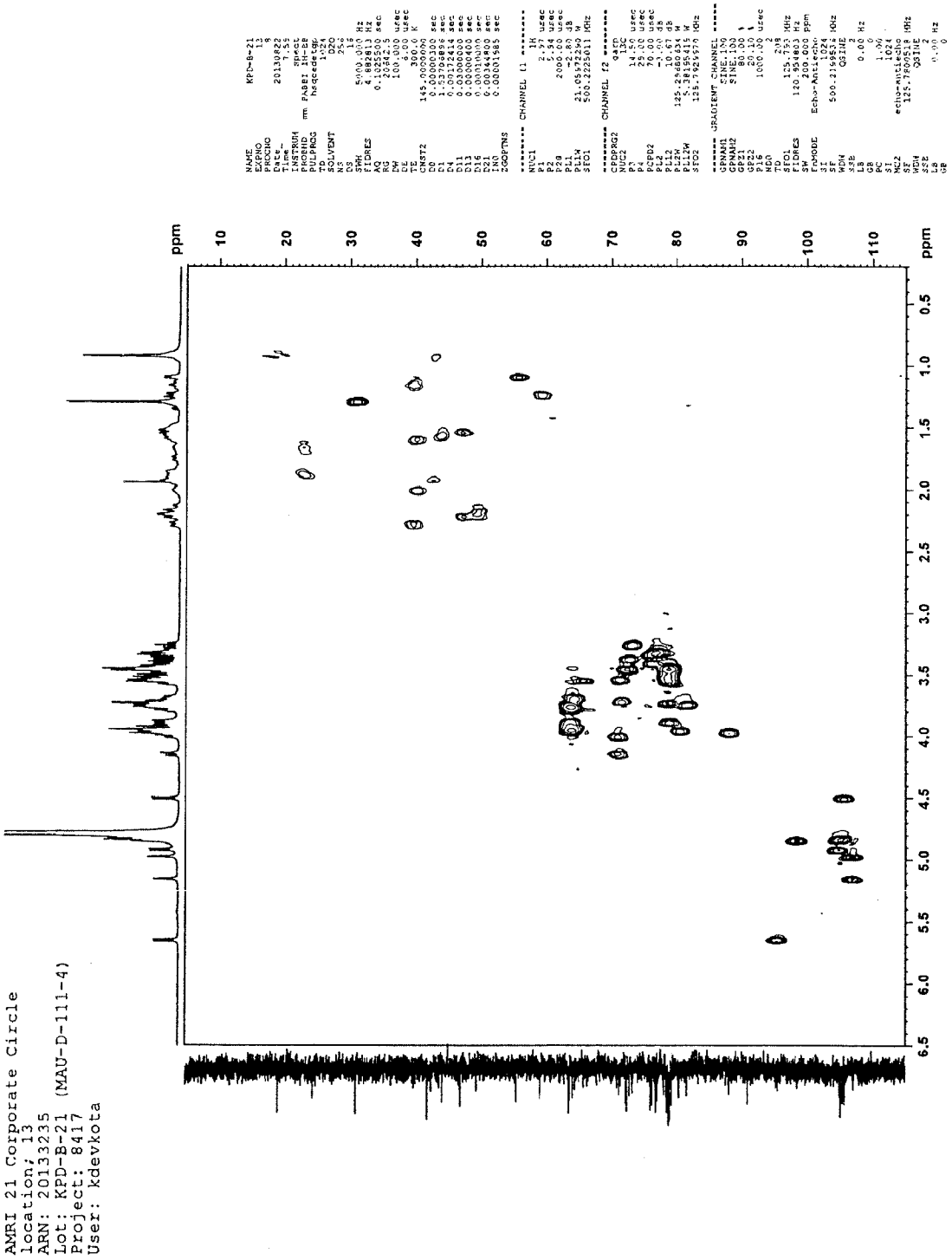

AMRI 21 Corporate Circle
location: 13
ARN: 20133235
Lot: KPD-B-21 (MAU-D-111-4)
Project: 8417
User: kdevkota
ppm

FIG. 34

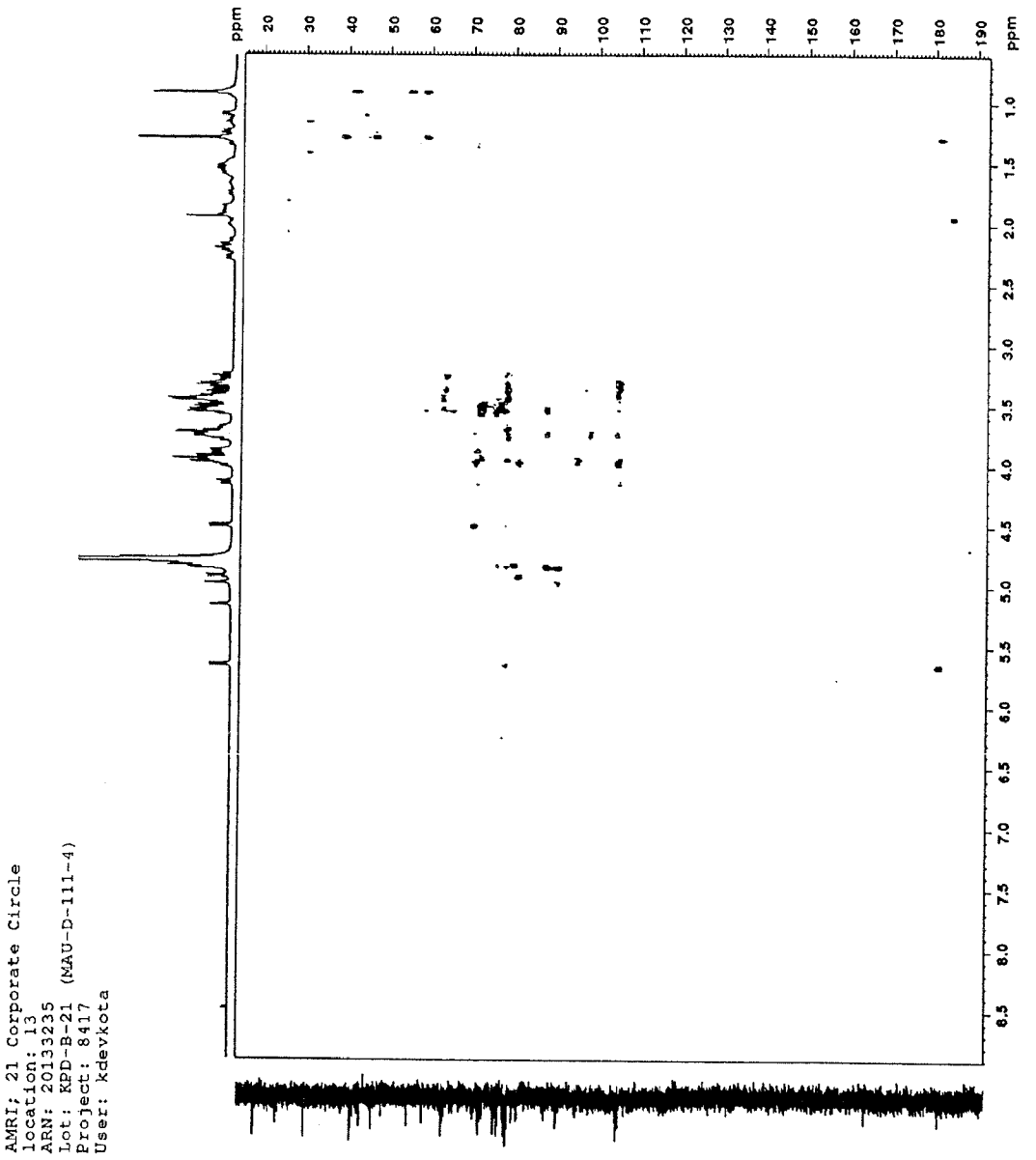

AMRI; 21 Corporate Circle
location: 13
ARN: 20133235
Lot: KPD-B-21 (MAU-D-111-4)
Project: 8417
User: kdevkota
ppm

FIG. 35

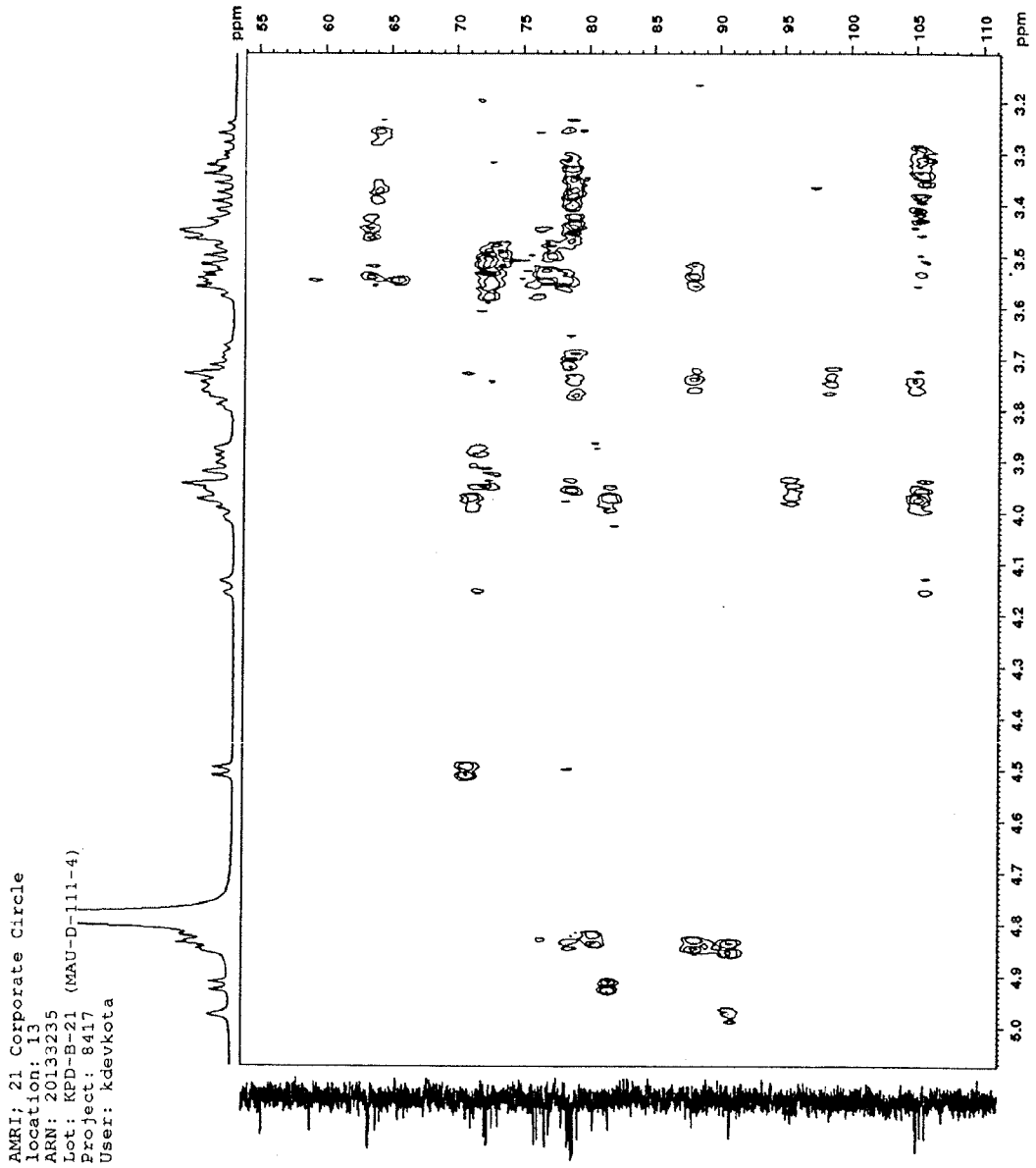

AMRI; 21 Corporate Circle
location: 13
ARN: 20133235
Lot: KPD-B-21 (MAU-D-111-4)
Project: 8417
User: kdevkota
ppm
ppm

FIG. 36

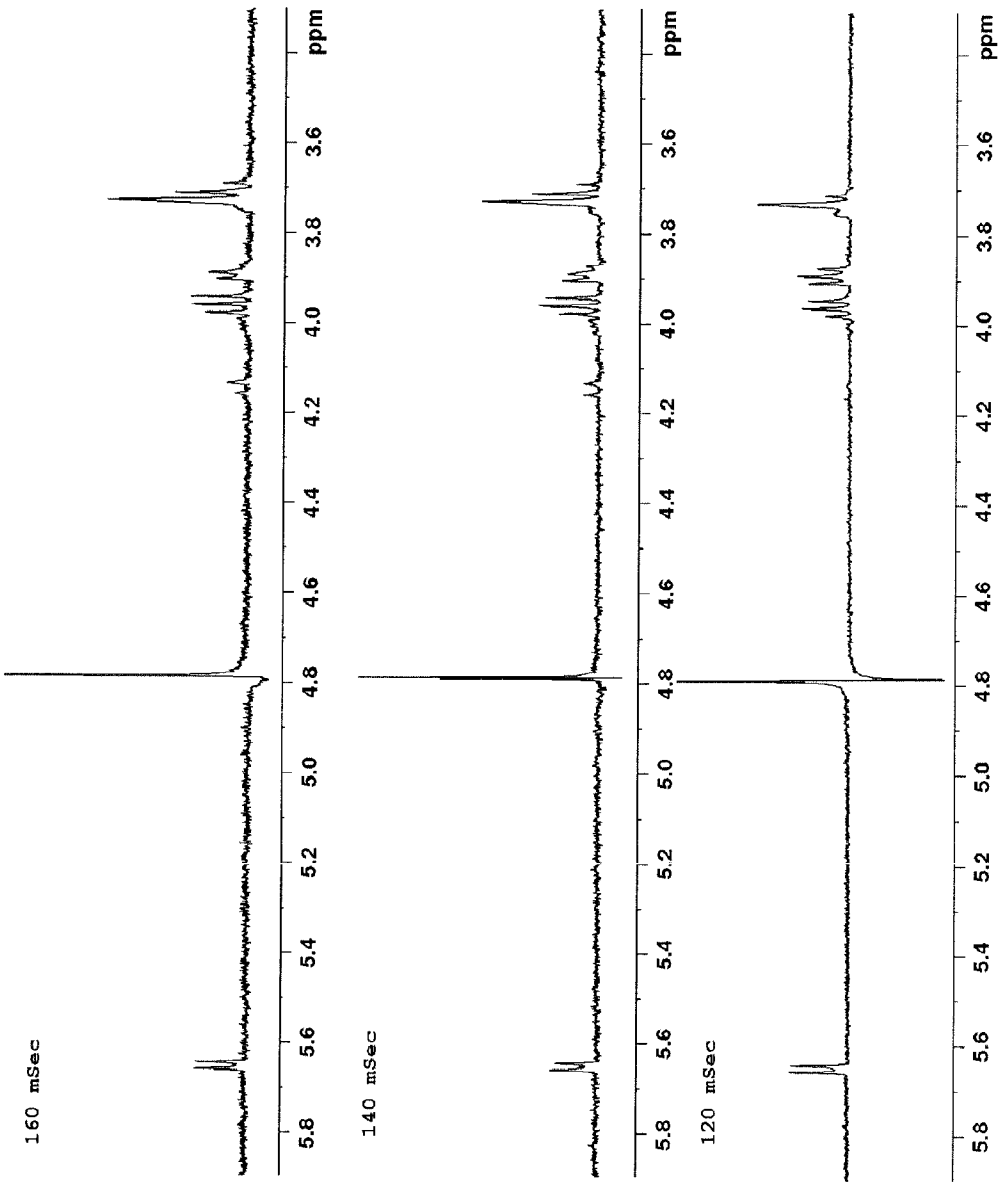


FIG. 37

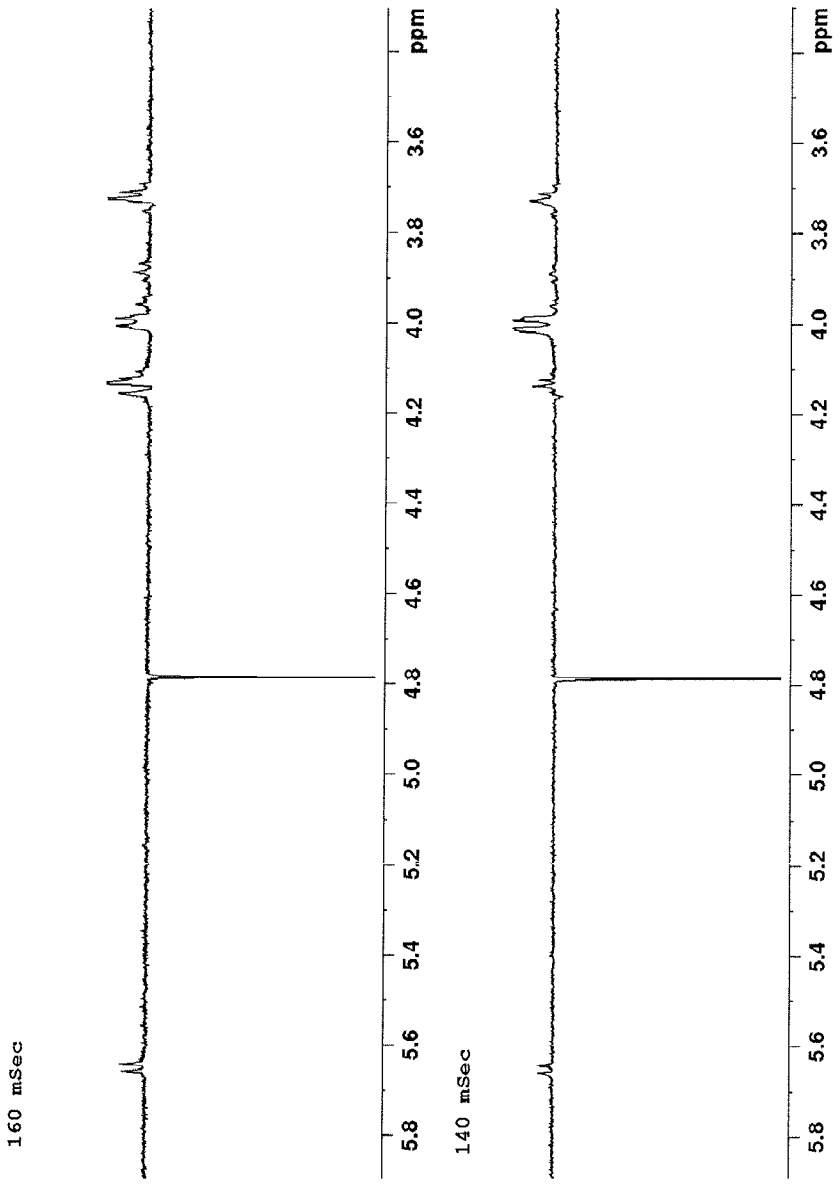


FIG. 38

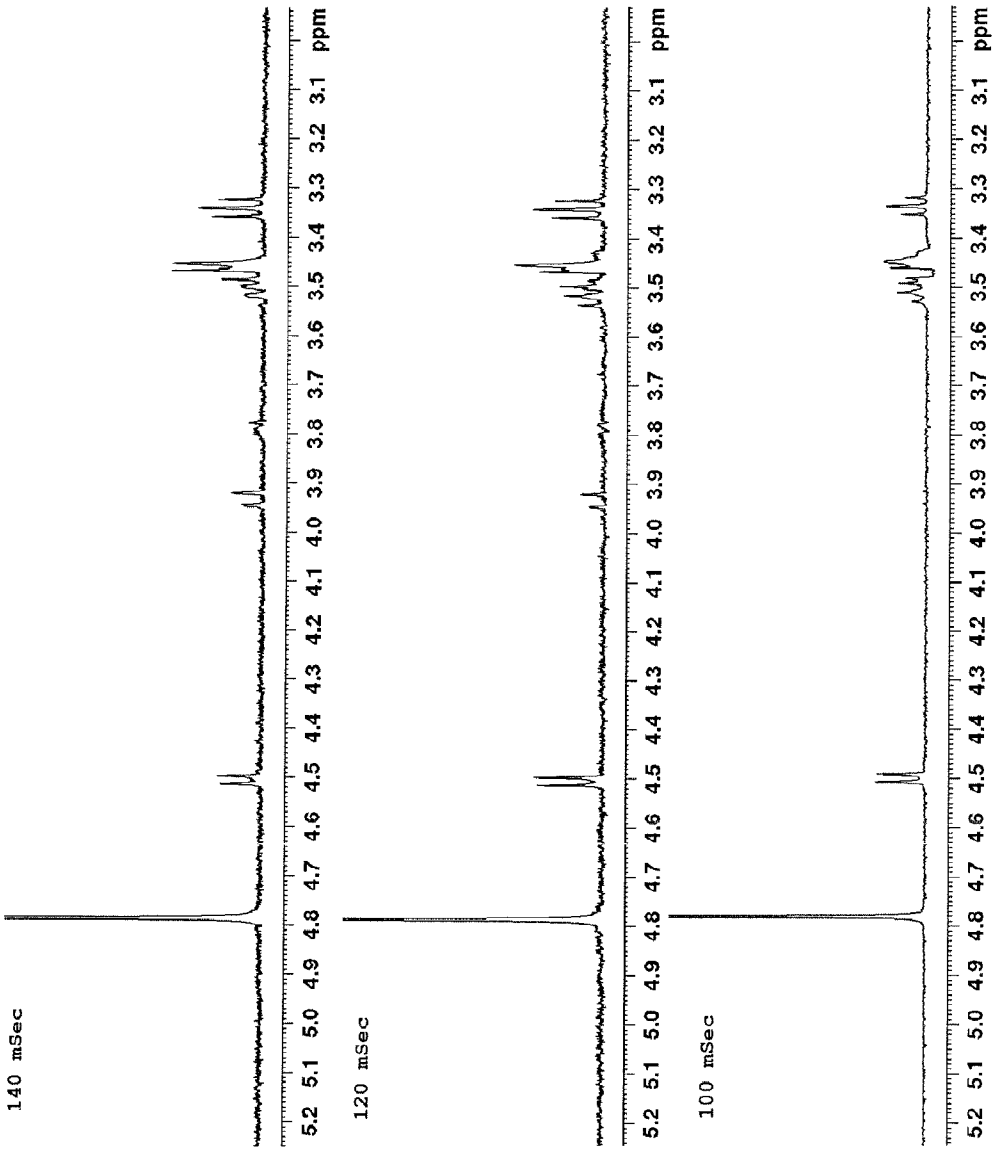

140 mSec
120 mSec
100 mSec
5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 ppm

FIG. 39

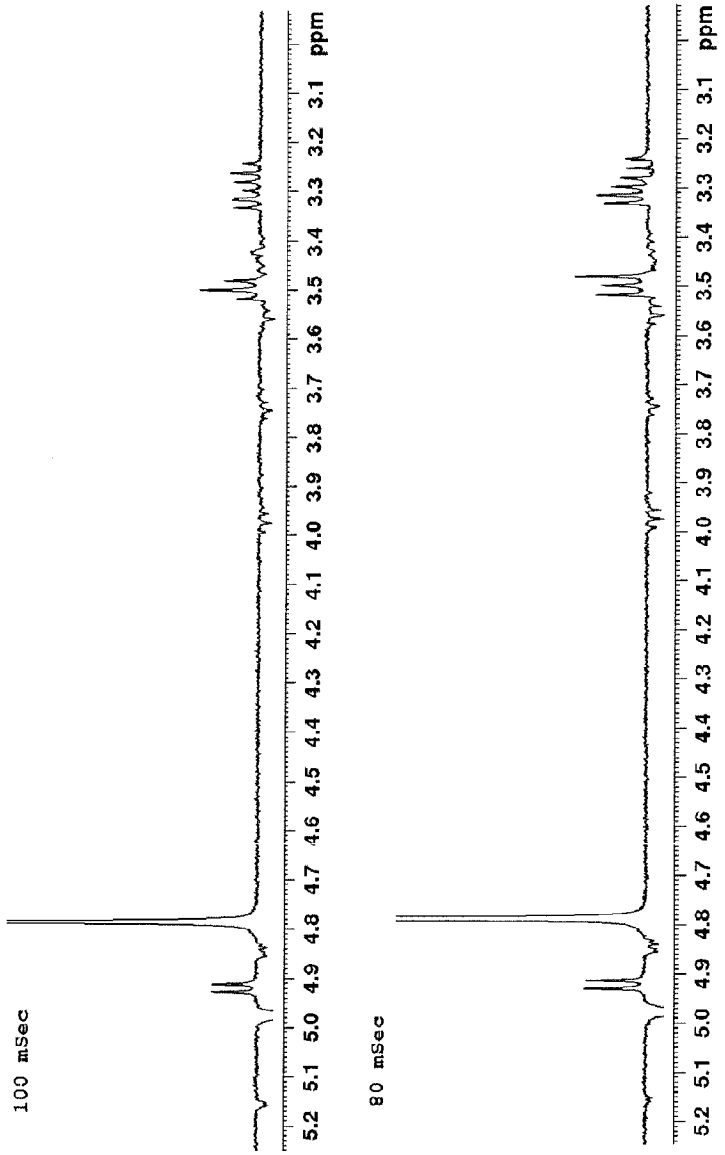
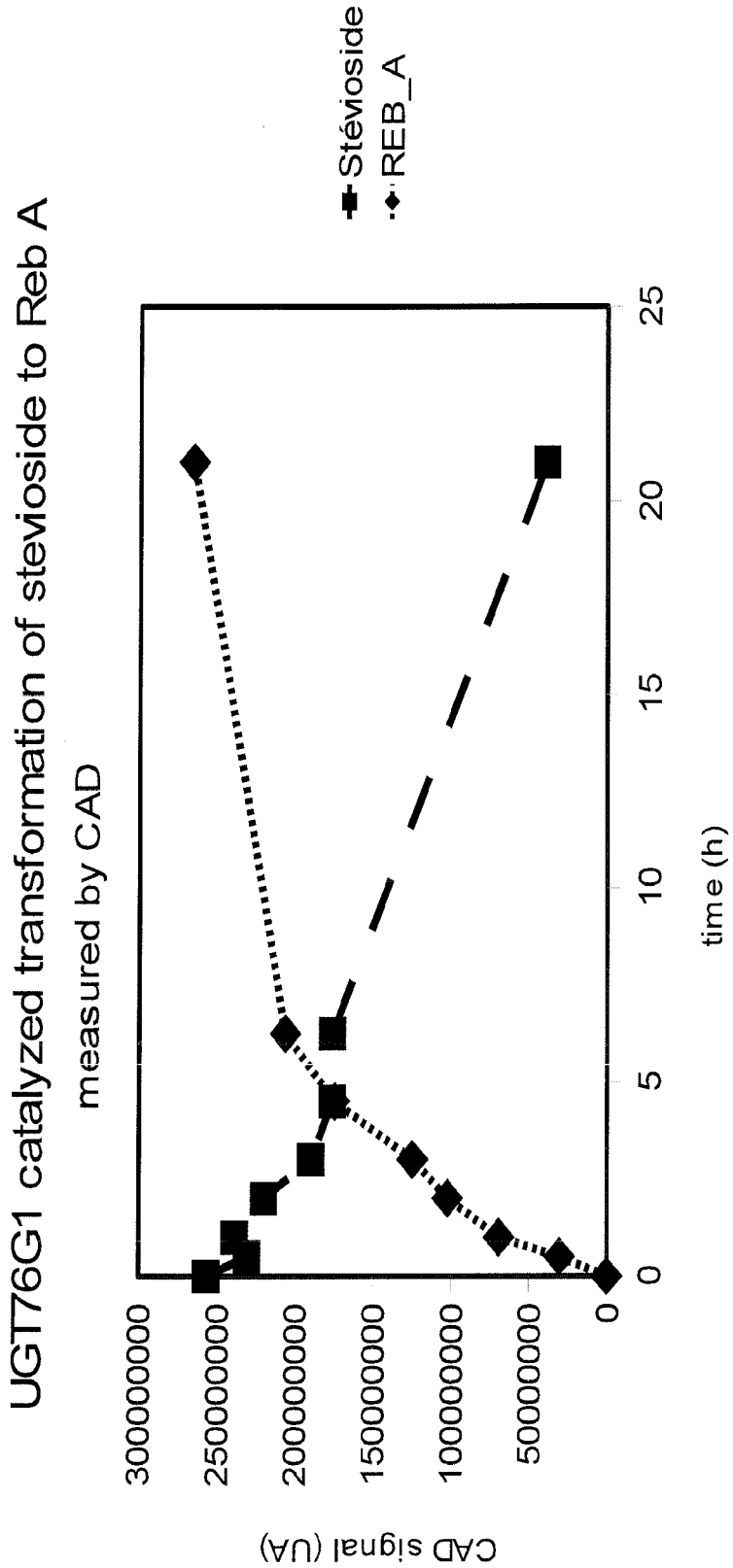

100 mSec
80 mSec
5.2 5.1 5.0 4.9 4.8 4.7 4.6 4.5 4.4 4.3 4.2 4.1 4.0 3.9 3.8 3.7 3.6 3.5 3.4 3.3 3.2 3.1 ppm

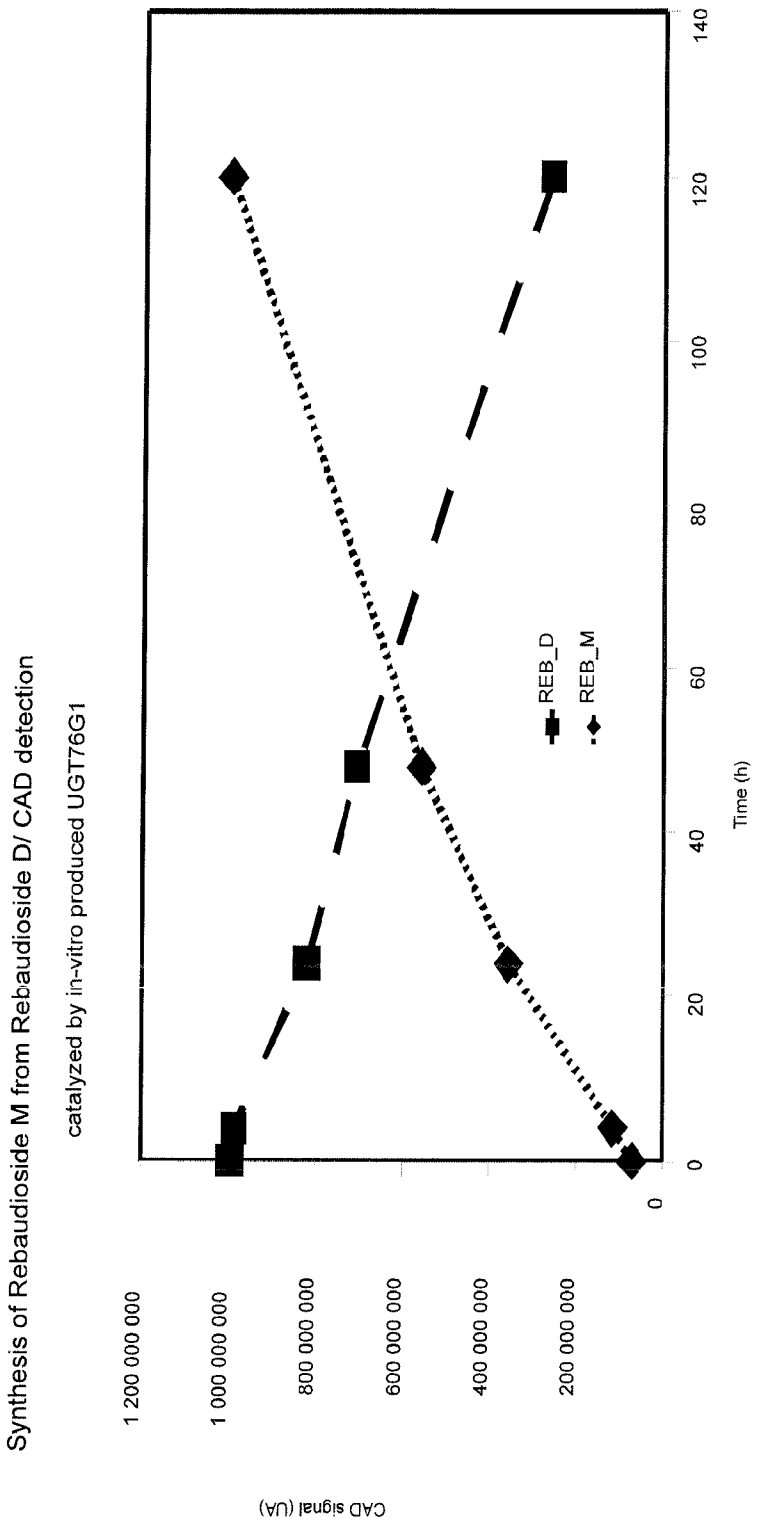
FIG. 40



Example 4

Example 6

FIG. 41



Example 20

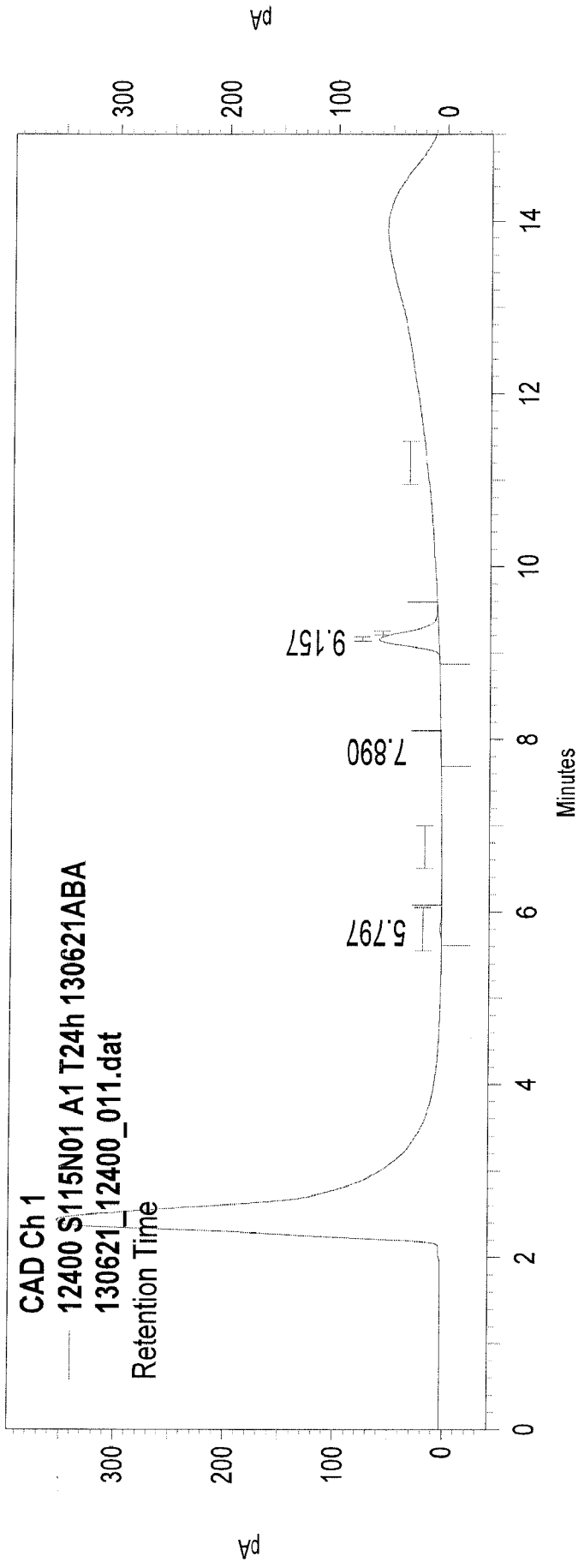


FIG. 42

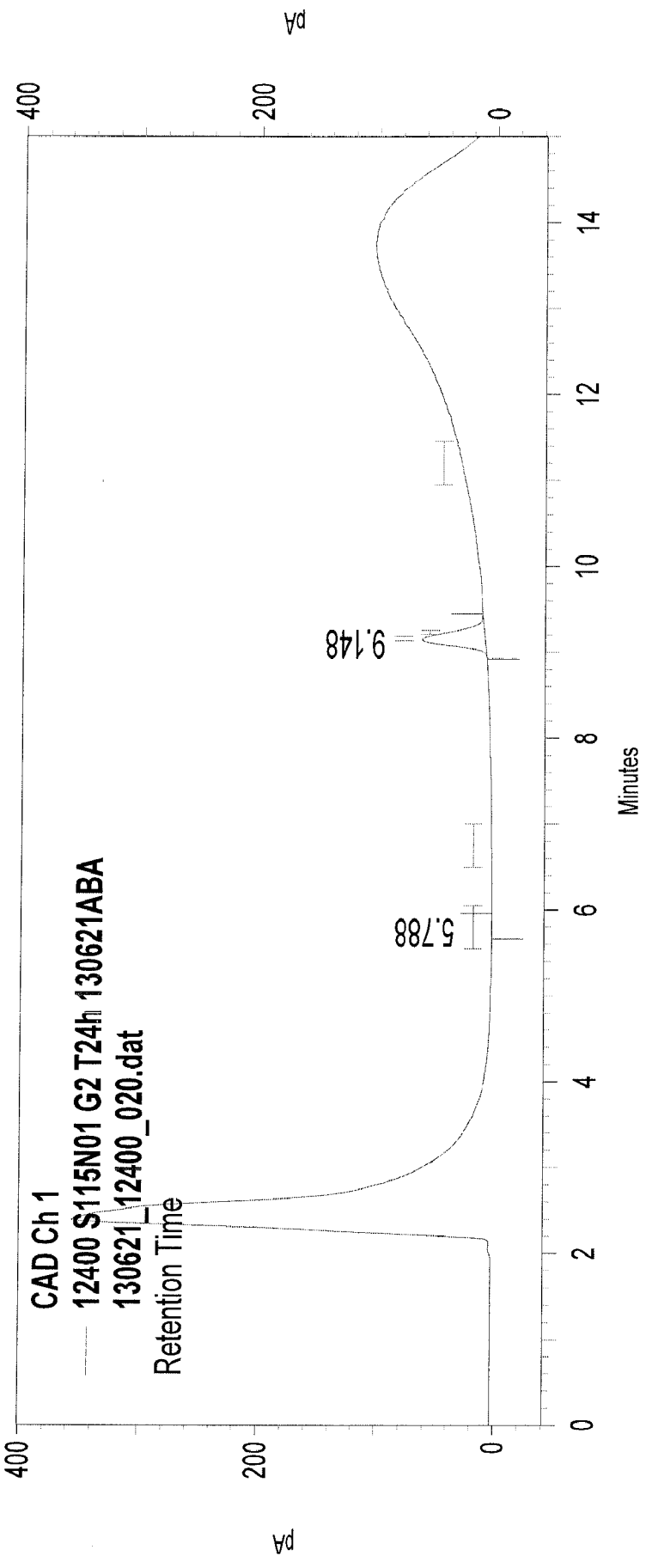

pA
400
200
0
CAD Ch 1
12400 S115N01 G2 T24h 130621ABA
130621_12400_020.dat
Retention Time
9.148
5.788
0
2
4
6
8
10
12
14
Minutes

FIG. 43

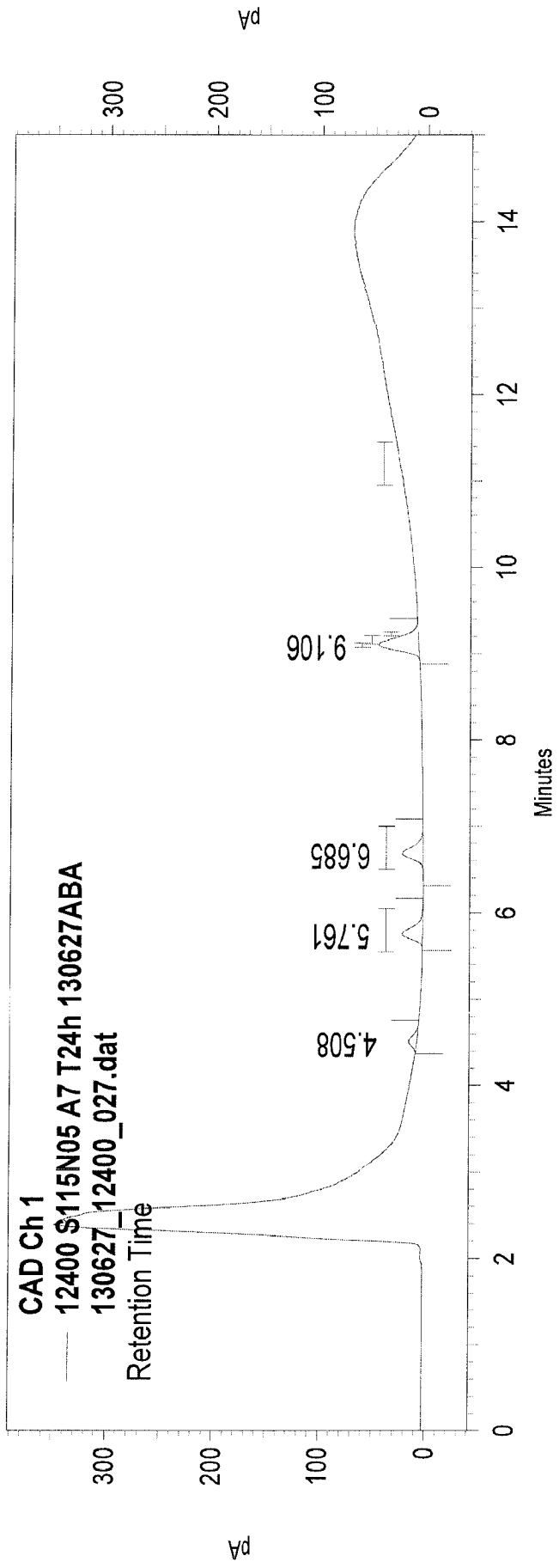

pA
300
200
100
0
CAD Ch 1
12400 S115N05 A7 T24h 130627ABA
130627_12400_027.dat
Retention Time
4.508
5.761
6.685
9.106
0
2
4
6
8
10
12
14
Minutes

FIG. 44

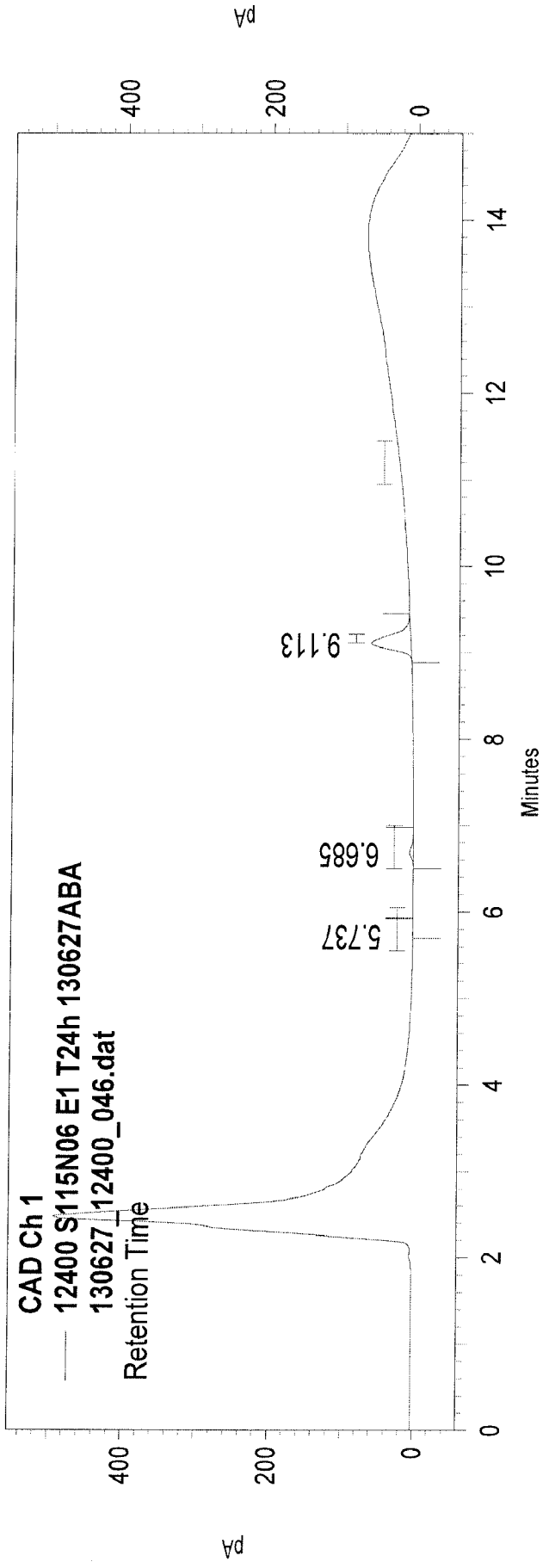
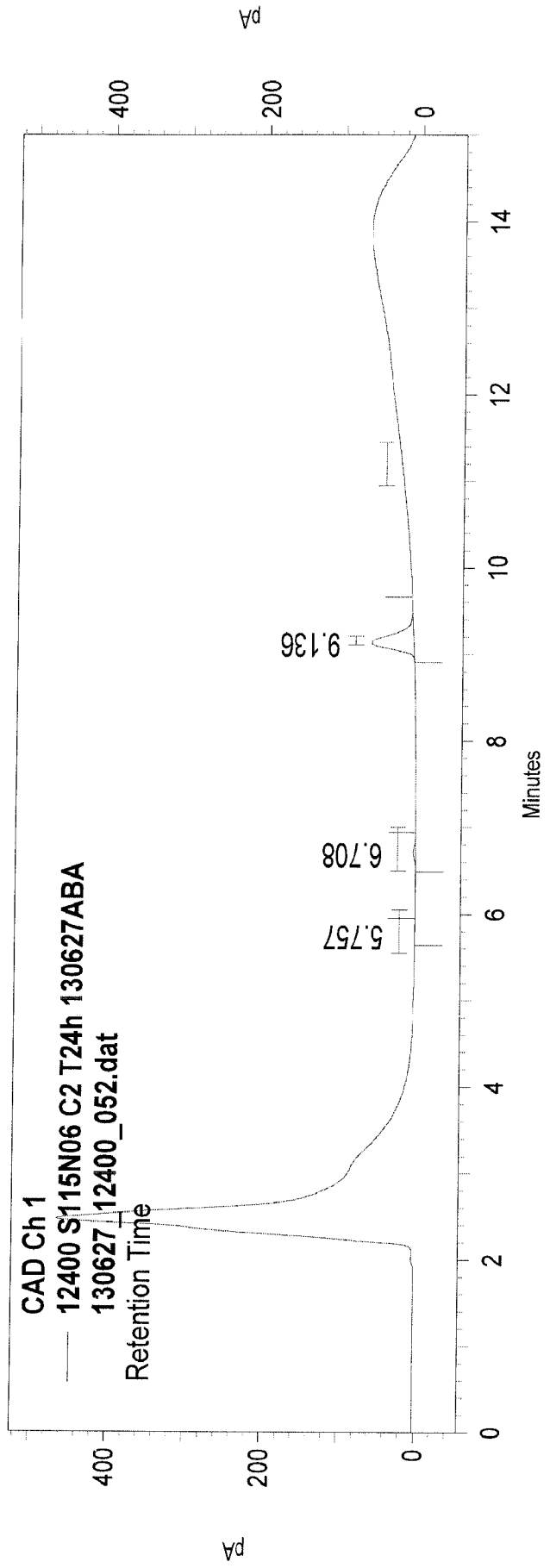

pA
400
200
0
CAD Ch 1
12400 S115N06 E1 T24h 130627ABA
130627_12400_046.dat
Retention Time
9.113
6.685
5.737
0
2
4
6
8
10
12
14
Minutes
pA
400
200
0

FIG. 45

FIG. 46


CAD Ch 1
12400 S115N06 C2 T24h 130627ABA
130627_12400_052.dat
Retention Time
9.136
6.708
5.757
pA
Minutes
0
200
400
2
4
6
8
10
12
14

Example 21

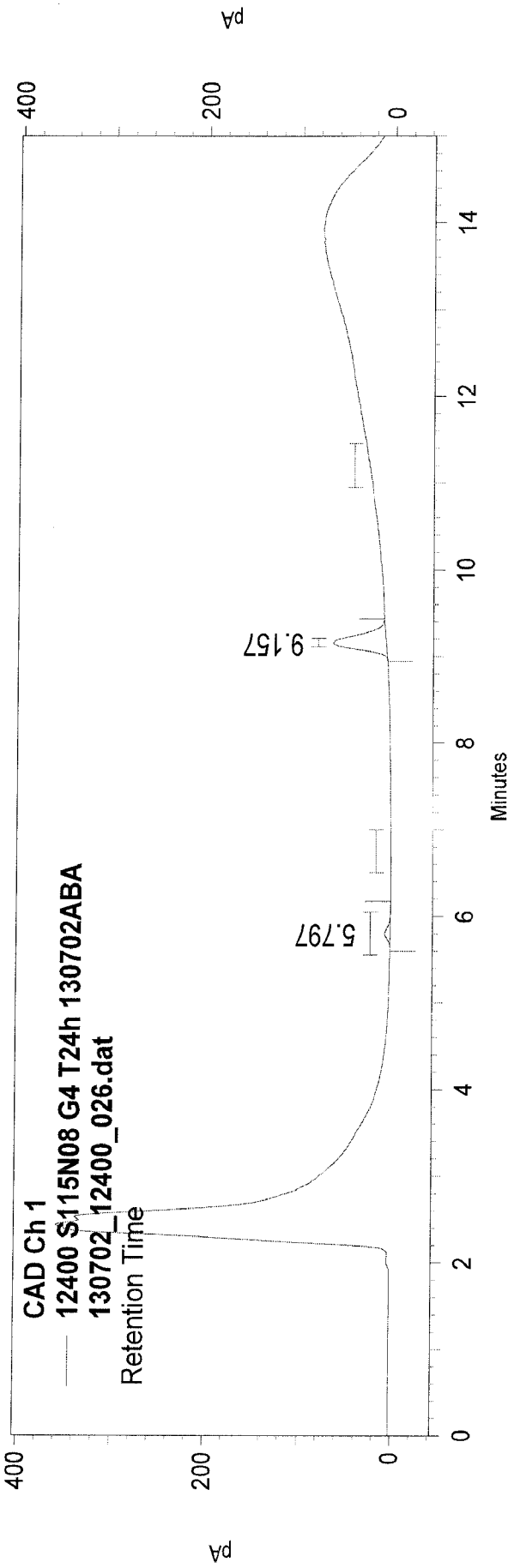


FIG. 47

Example 22

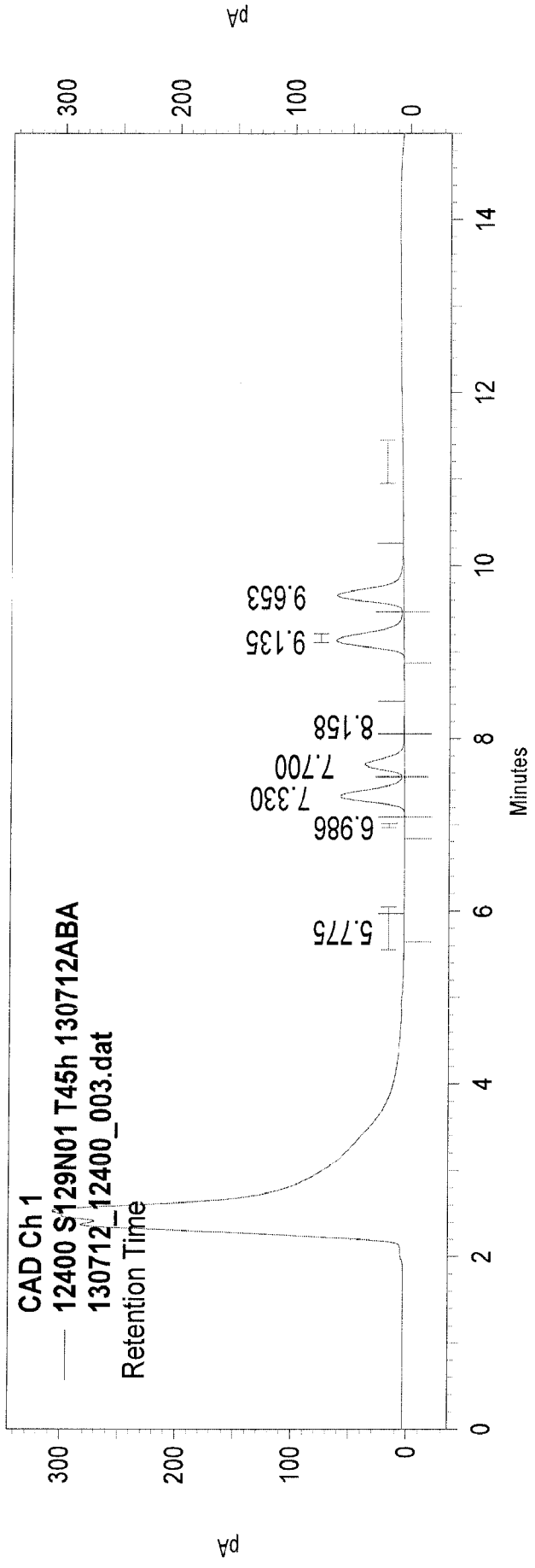


FIG. 48

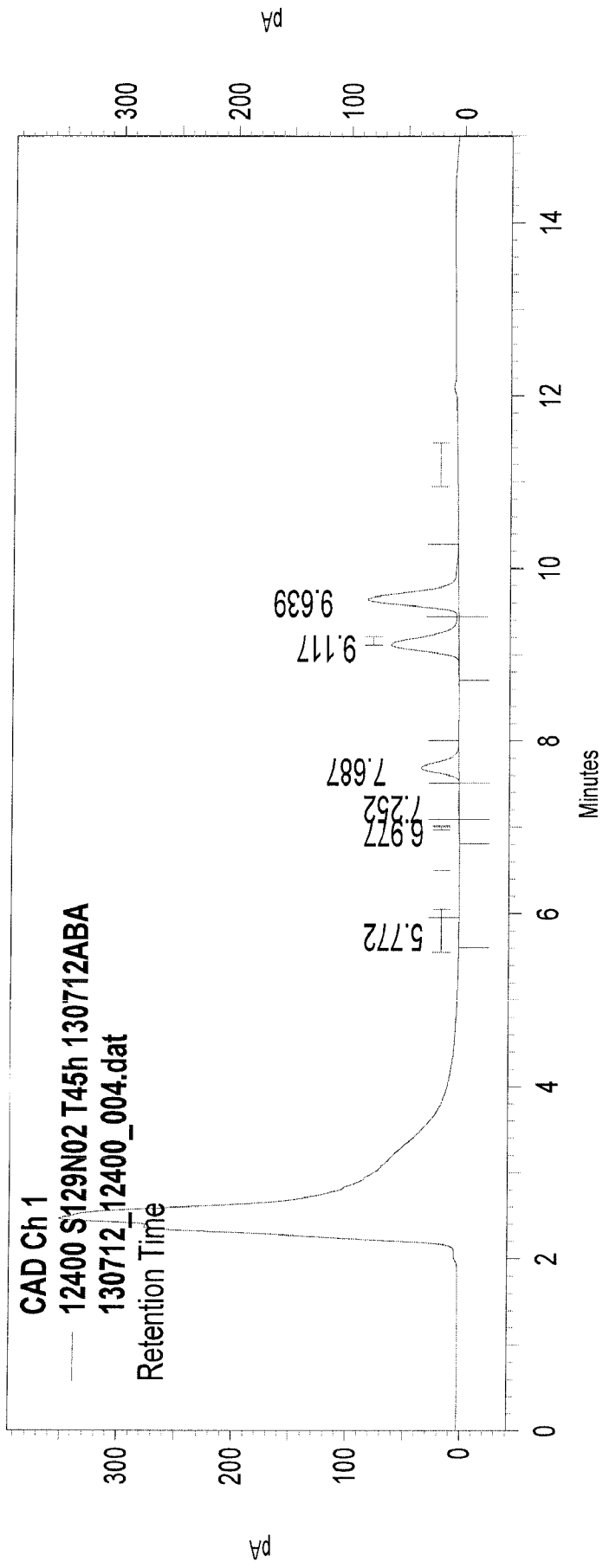
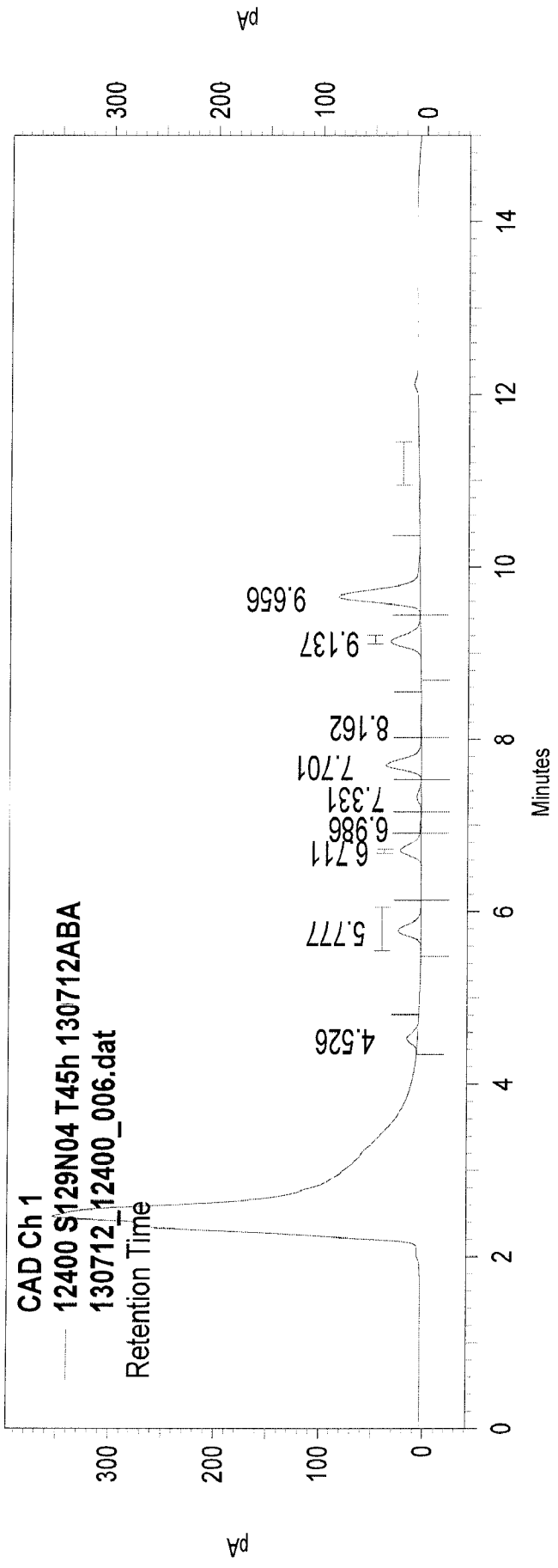

pA
300
200
100
0
CAD Ch 1
12400 S129N02 T45h 130712ABA
130712_12400_004.dat
Retention Time
5.772
6.977
7.252
7.687
9.117
9.639
0
2
4
6
8
10
12
14
Minutes

FIG. 49

FIG. 50


pA
300
200
100
0
CAD Ch 1
12400 S129N04 T45h 130712ABA
130712_12400_006.dat
Retention Time
4.526
5.777
6.711
6.986
7.331
7.701
8.162
9.137
9.656
0
2
4
6
8
10
12
14
Minutes
pA
300
200
100
0

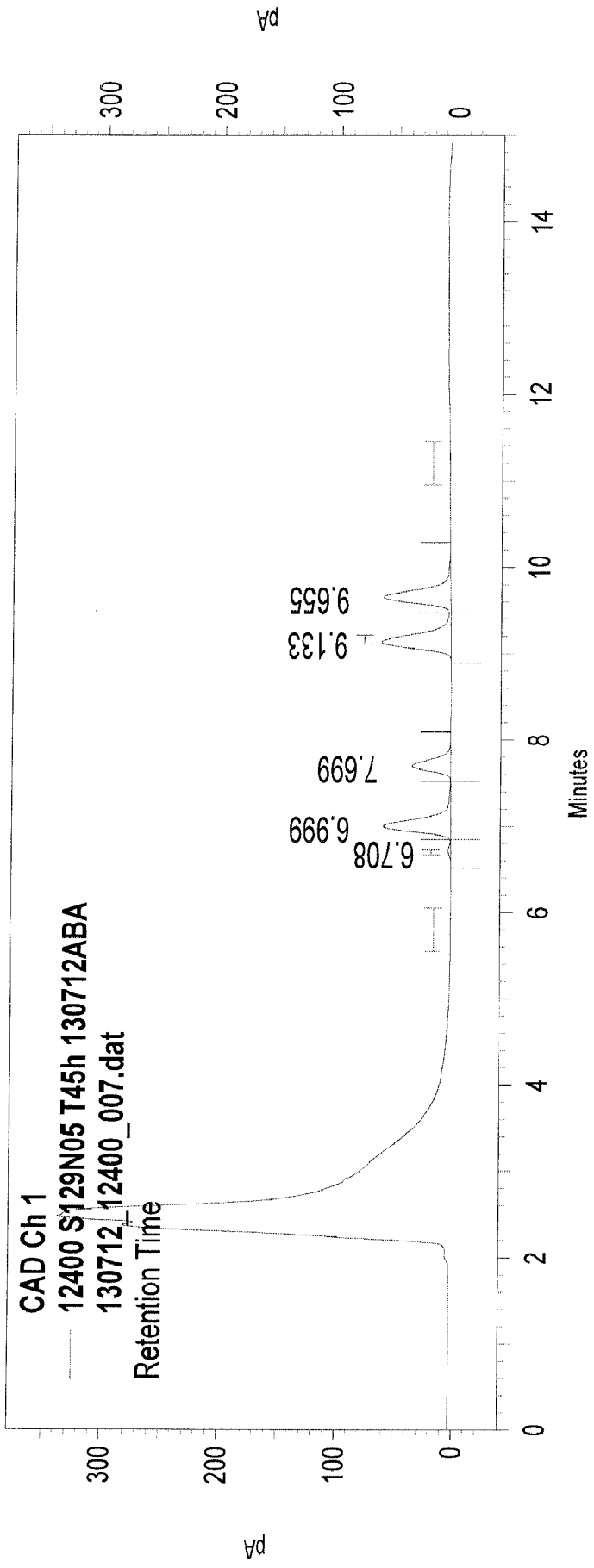

CAD Ch 1
12400 S129N05 T45h 130712ABA
130712_12400_007.dat
Retention Time
6.708
6.999
7.699
9.133
9.655
pA
Minutes
0
2
4
6
8
10
12
14
0
100
200
300

FIG. 51

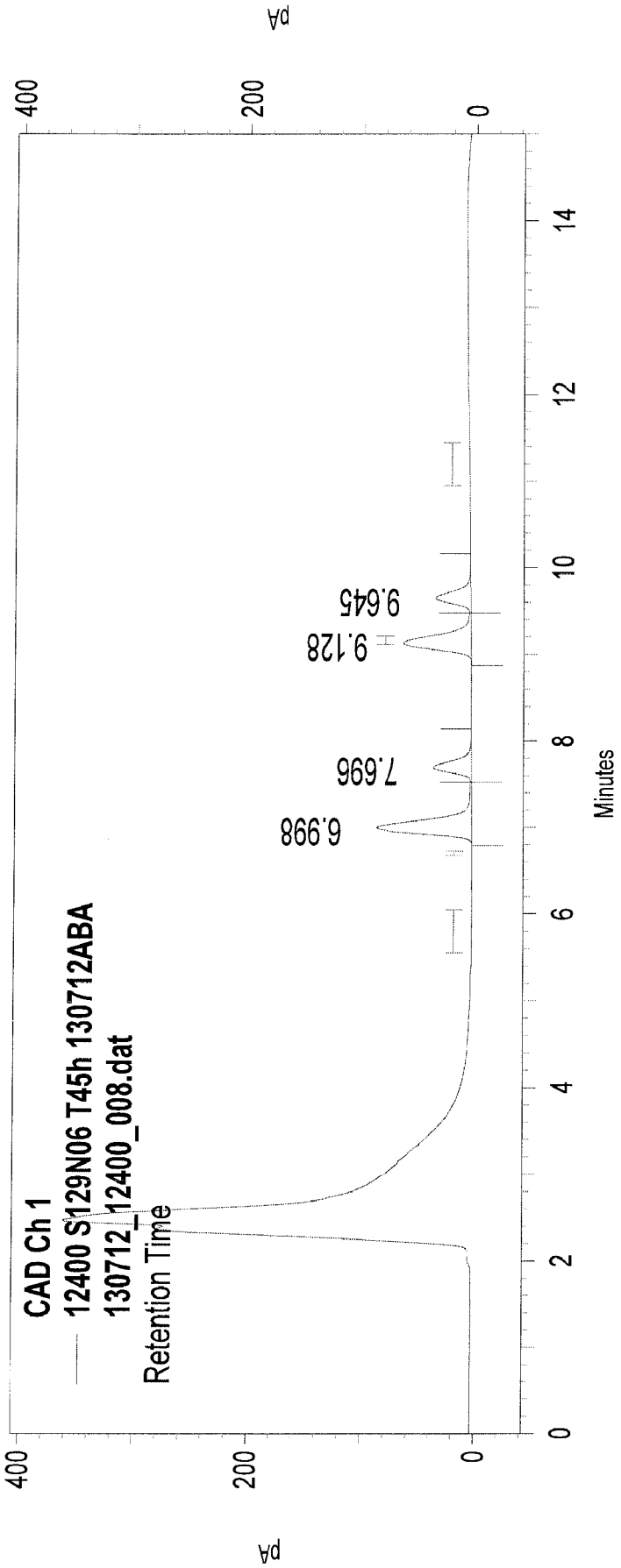

pA
400
200
0
CAD Ch 1
12400 S129N06 T45h 130712ABA
130712_12400_008.dat
Retention Time
6.998
7.696
9.128
9.645
0
2
4
6
8
10
12
14
Minutes
pA
400
200
0

FIG. 52

Example 23

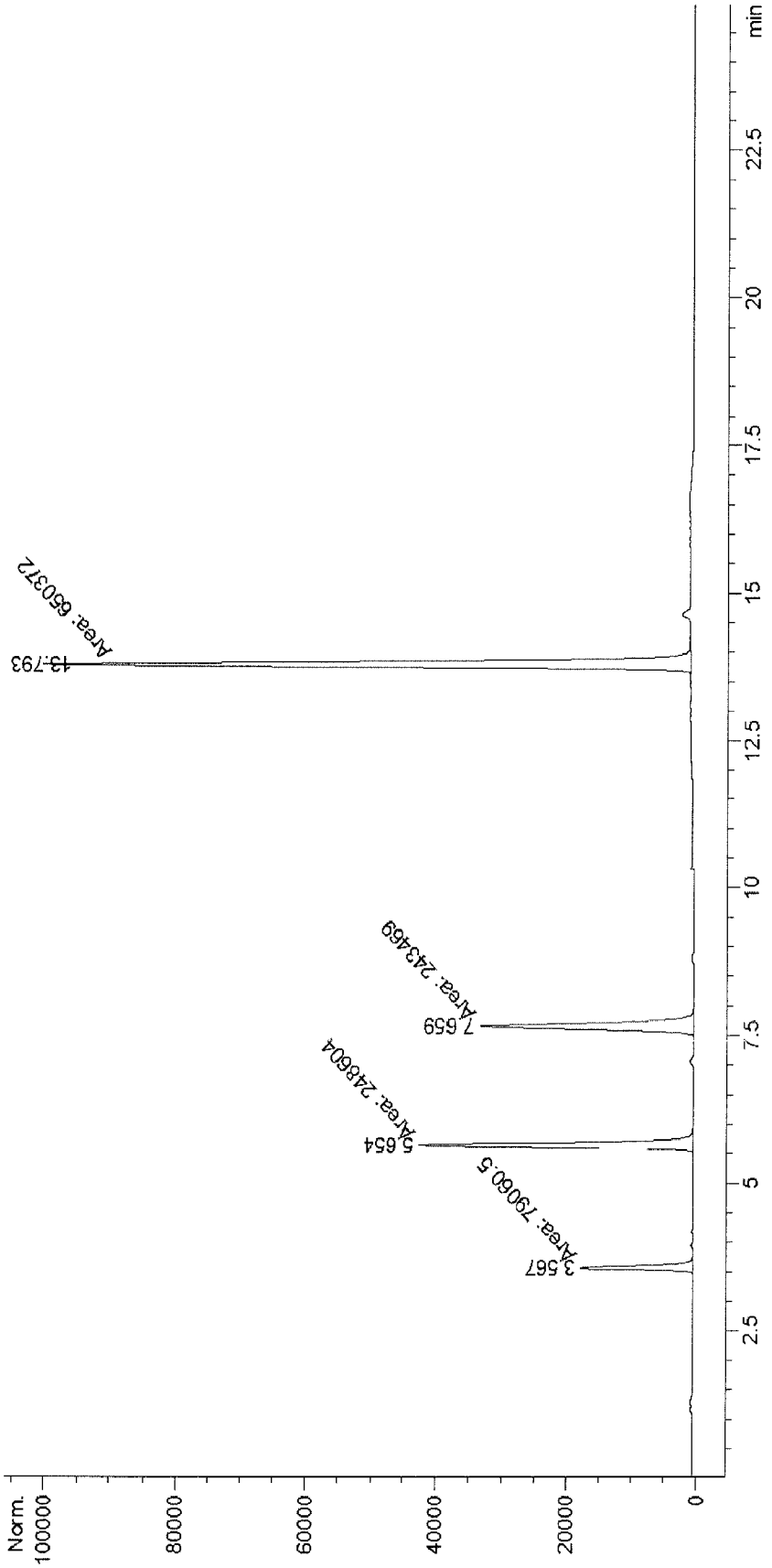
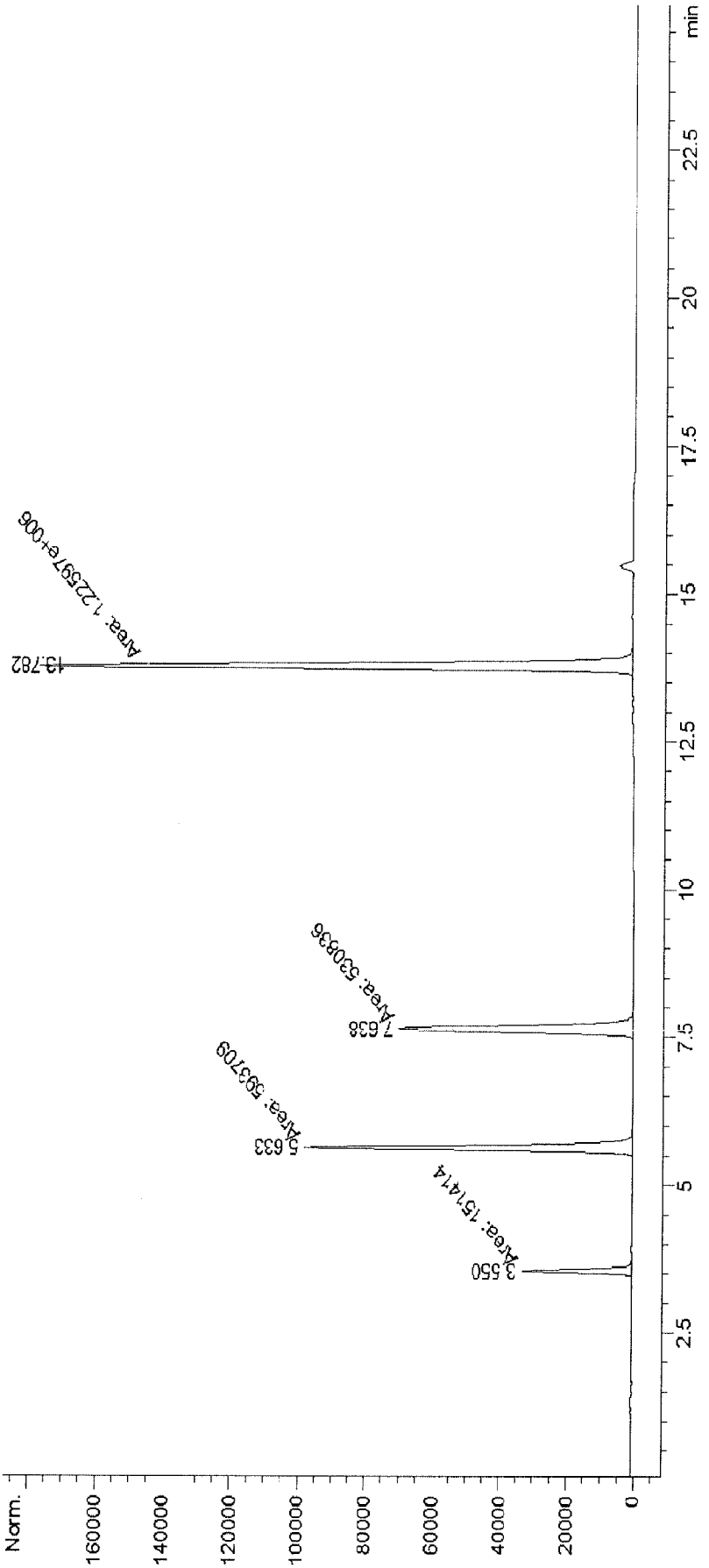


FIG. 53

FIG. 54


Norm.
160000
140000
120000
100000
80000
60000
40000
20000
0
2.5
5
7.5
10
12.5
15
17.5
20
22.5
min
3.550
Area: 151414
5.633
Area: 595709
7.638
Area: 530836
13.782
Area: 1.22597e+006

Example 24

FIG. 55

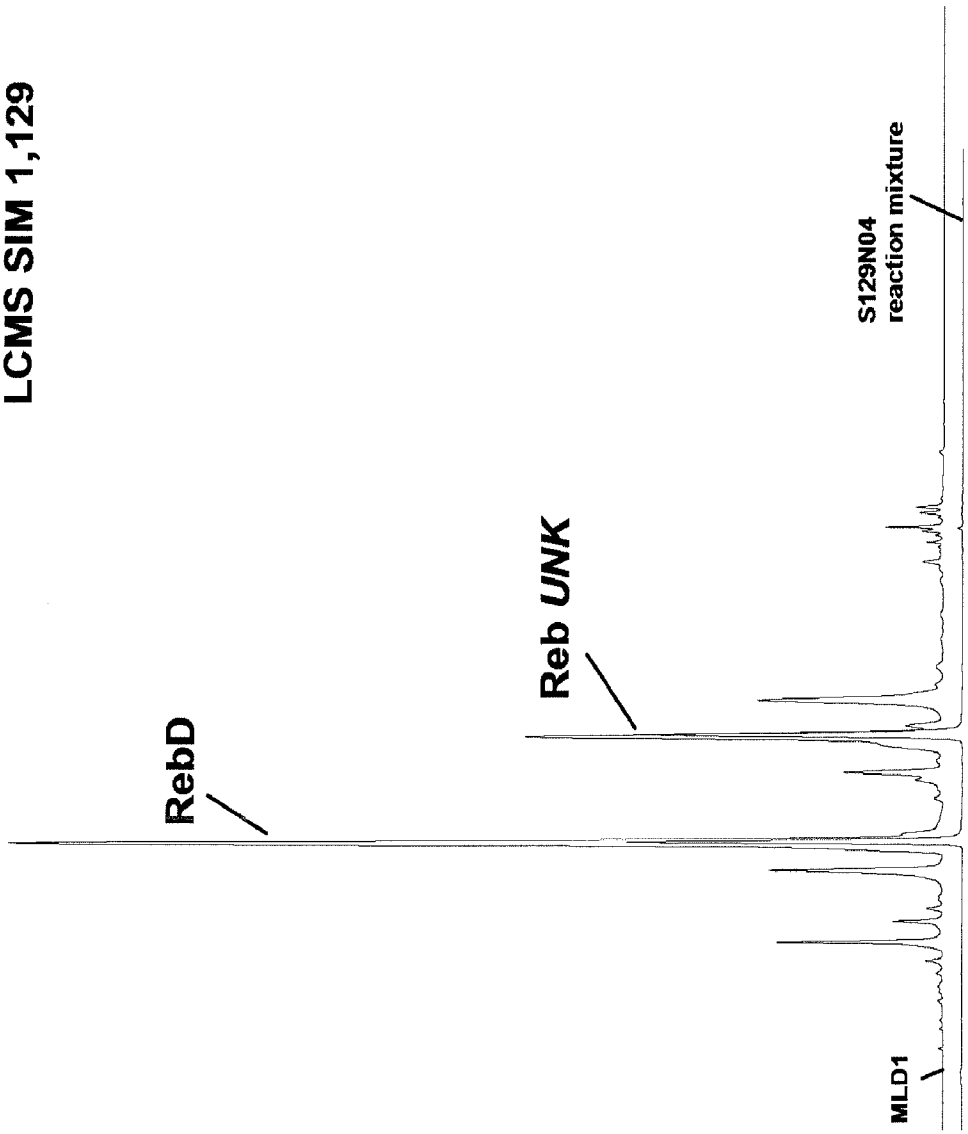
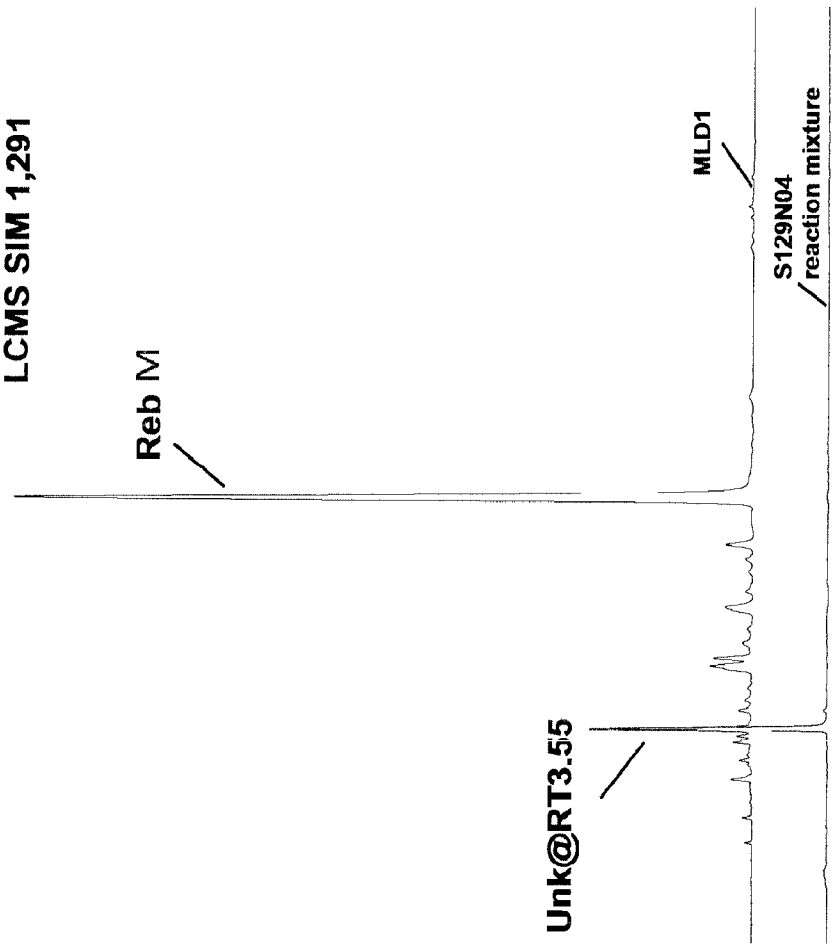


FIG. 56



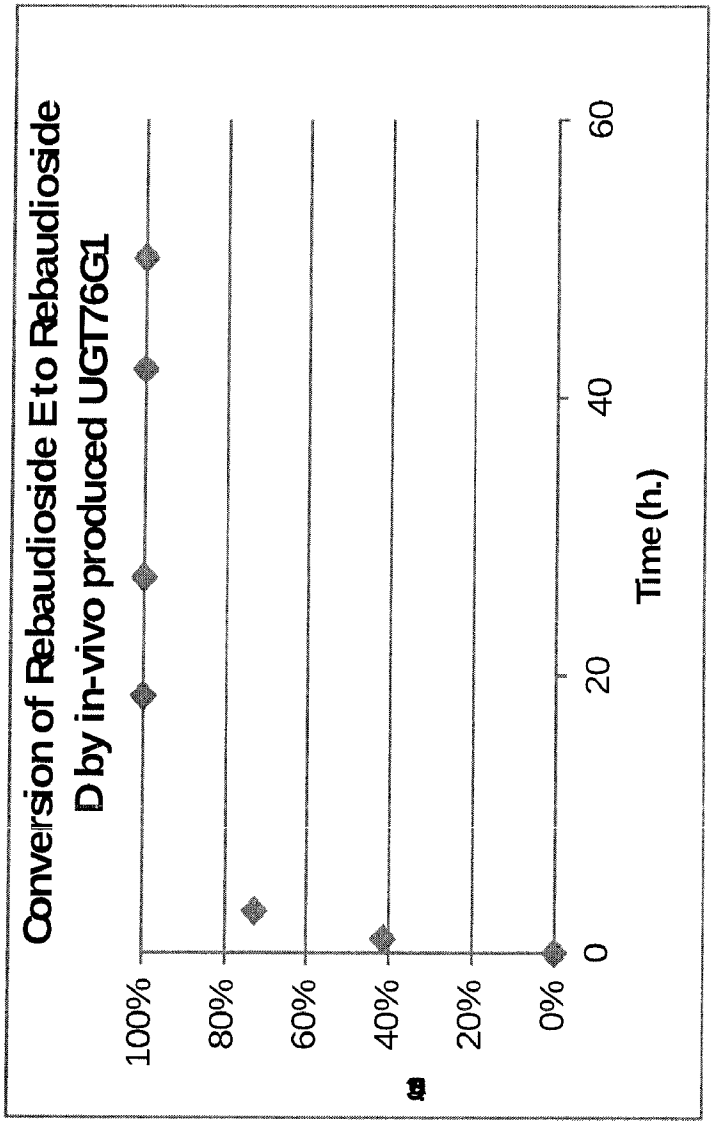


FIG. 57

Example 25

Example 28

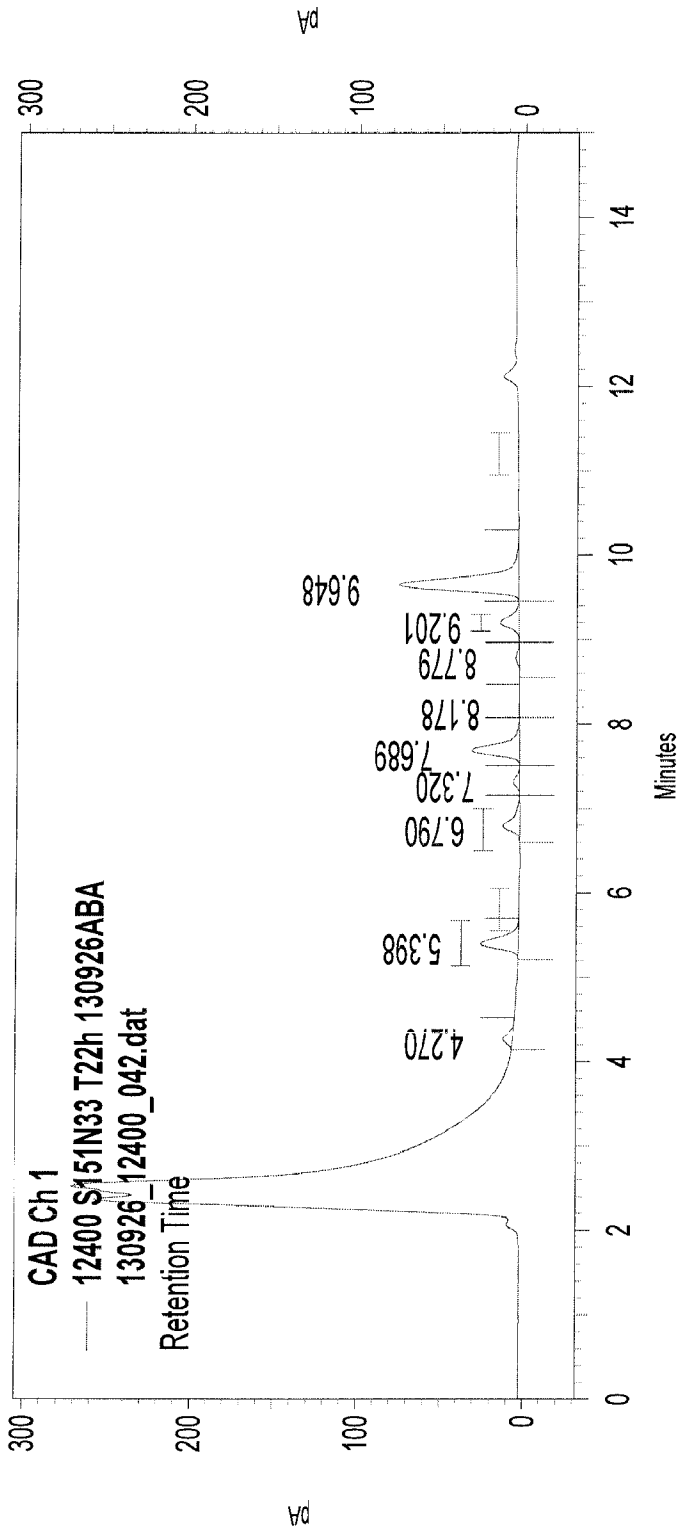
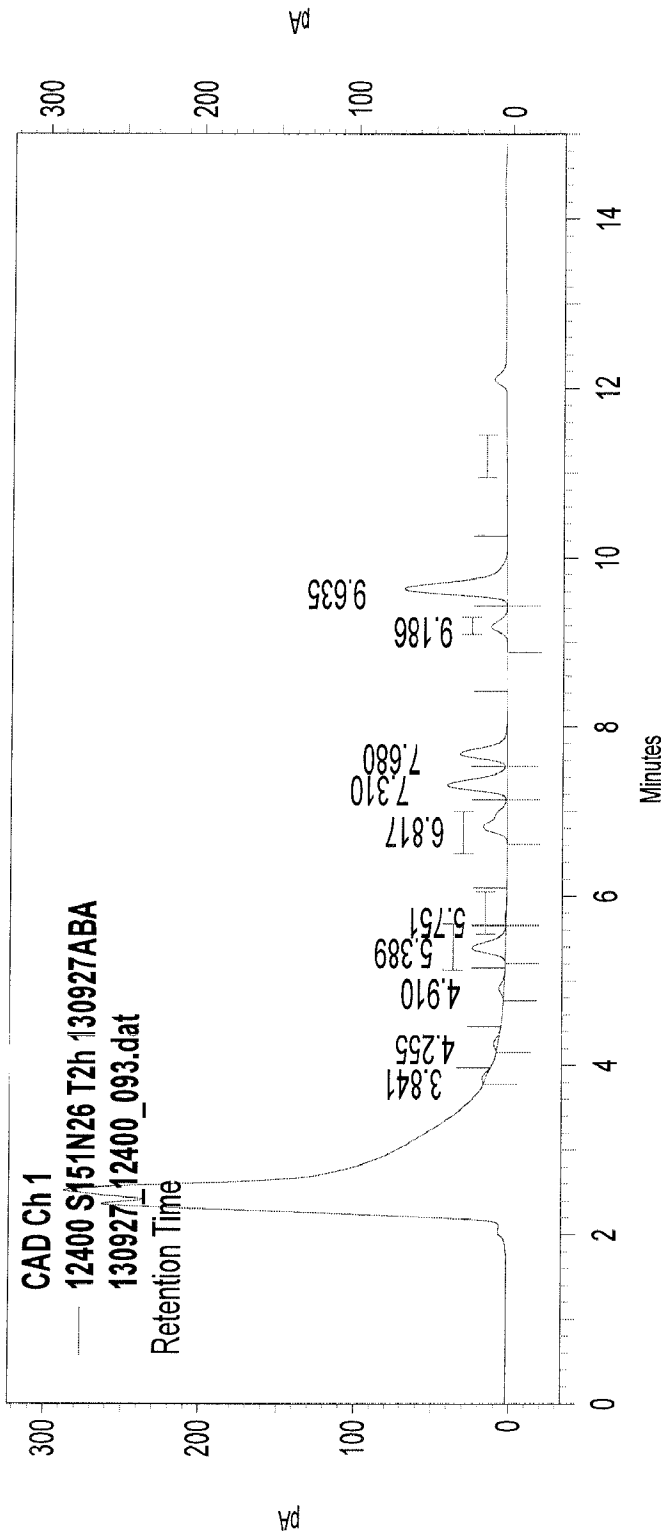


FIG. 58

FIG. 59


pA
300
200
100
0
CAD Ch 1
12400 S151N26 T2h 130927ABA
130927_12400_093.dat
Retention Time
3.841
4.255
4.910
5.389
5.751
6.817
7.310
7.680
9.186
9.635
0
2
4
6
8
10
12
14
Minutes

Example 29

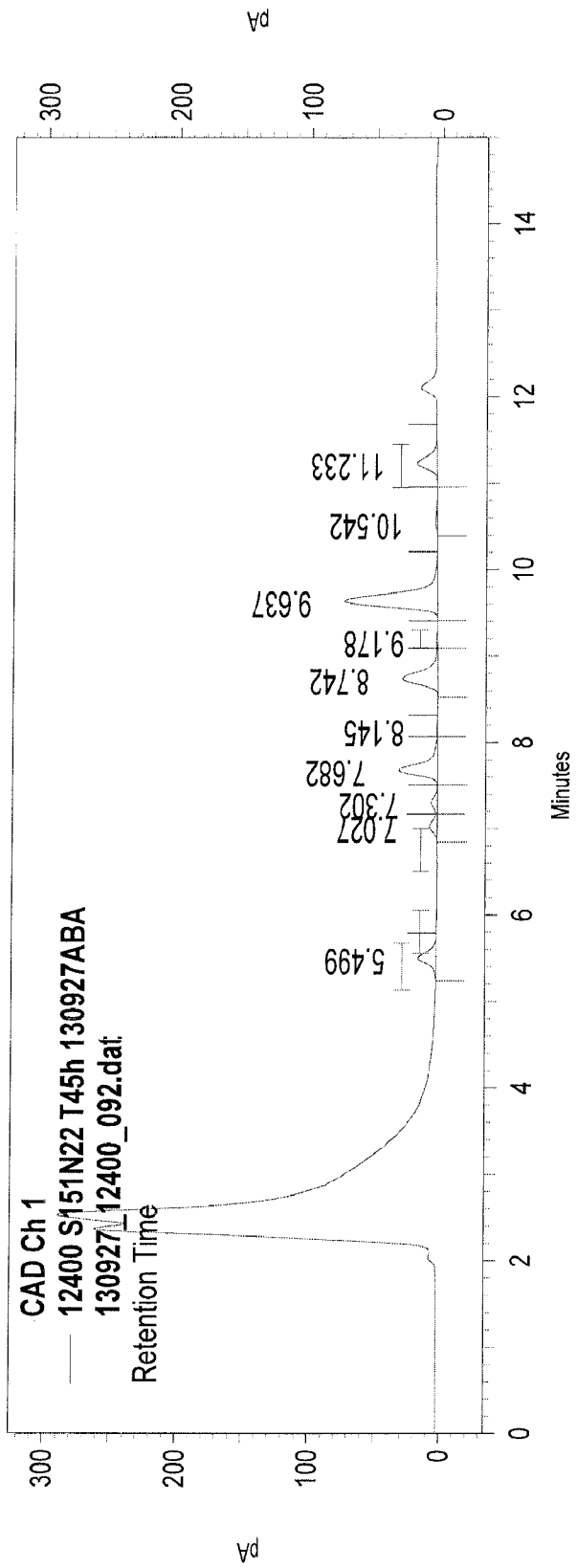
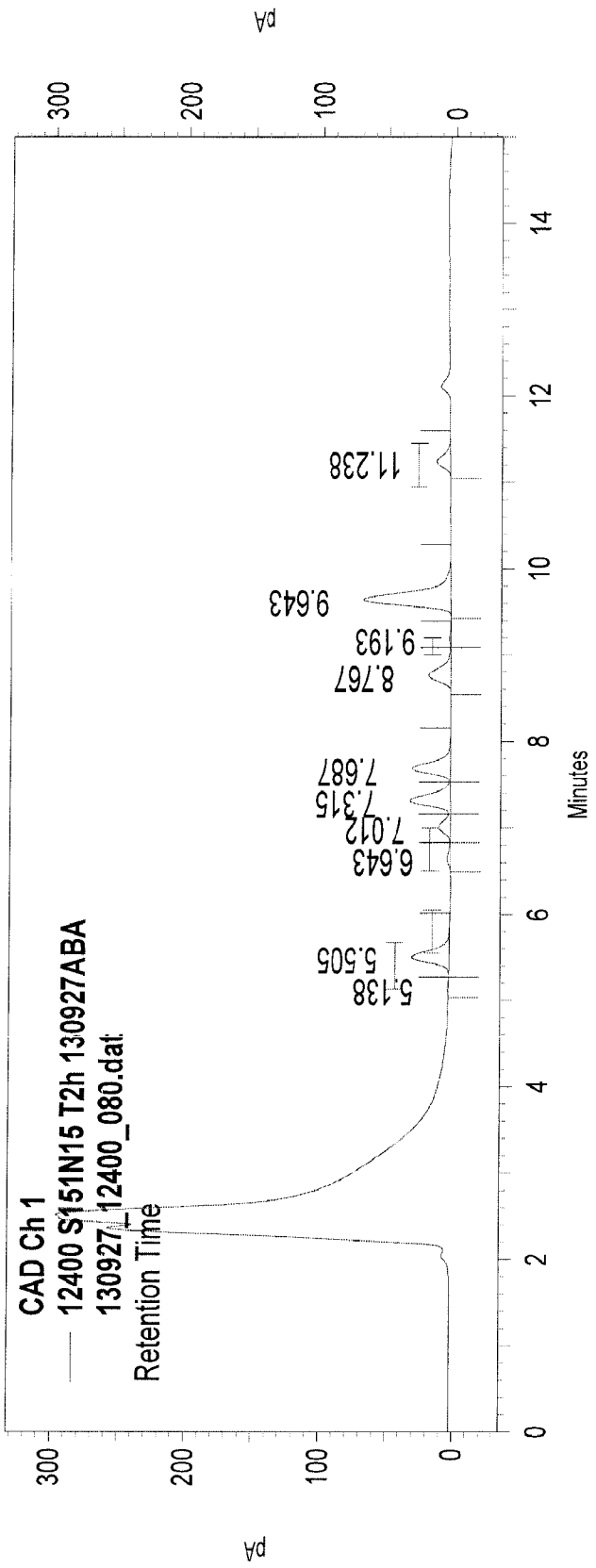


FIG. 60

FIG. 61


pA
300
200
100
0
CAD Ch 1
12400 S151N15 T2h 130927ABA
130927_12400_080.dat
Retention Time
5.138
5.505
6.643
7.012
7.315
7.687
8.767
9.193
9.643
11.238
0
2
4
6
8
10
12
14
Minutes

Example 30

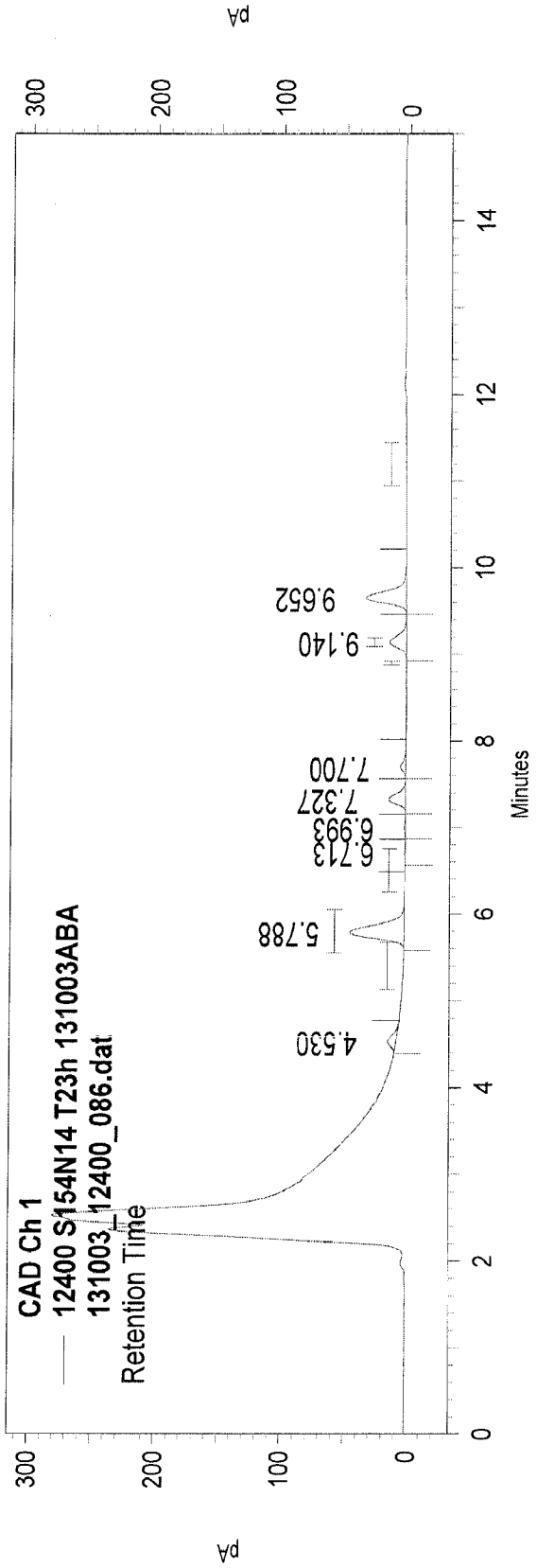
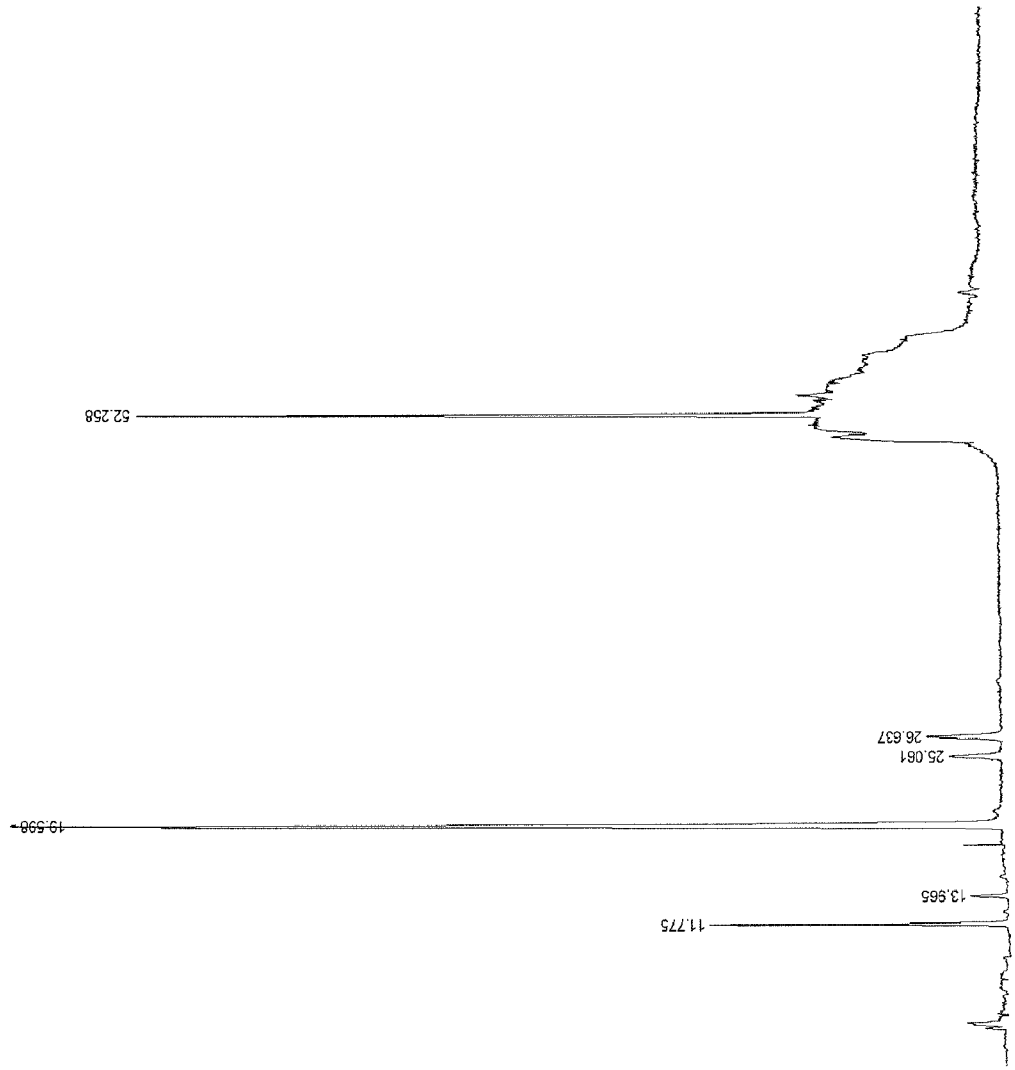


FIG. 62

Example 31 **FIG. 63**



Example 32

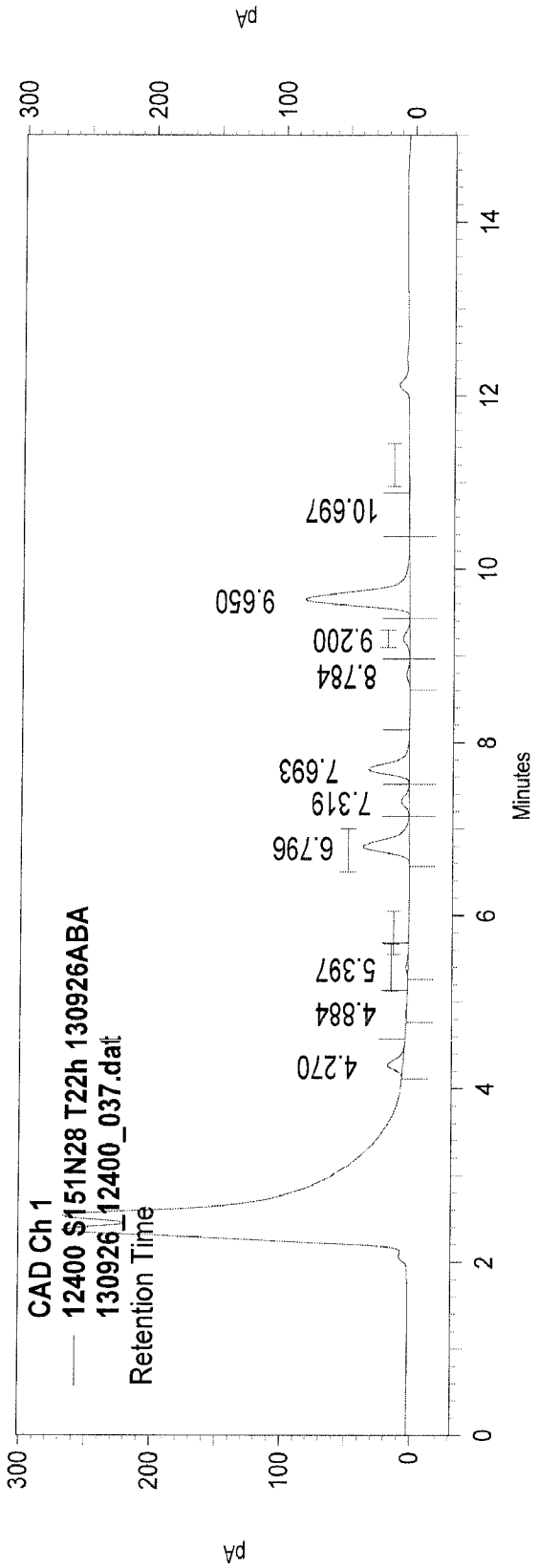


FIG. 64

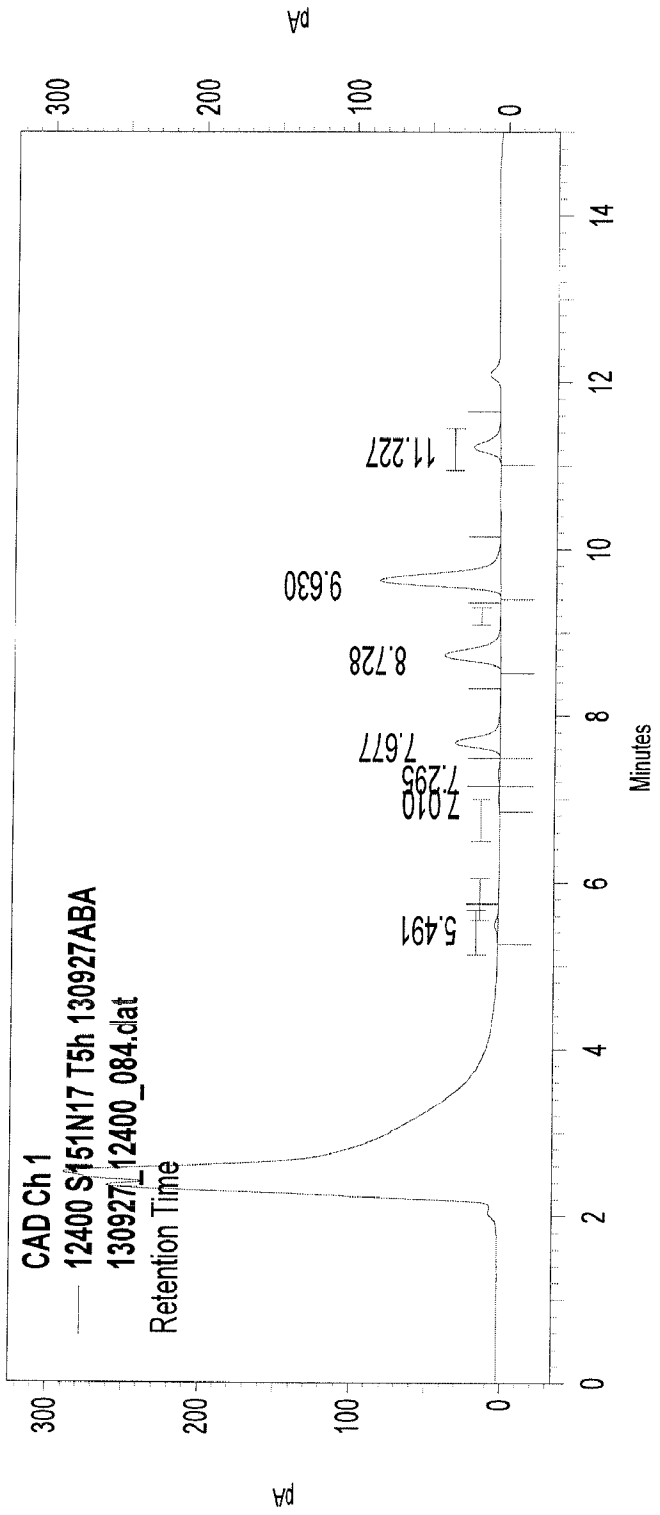
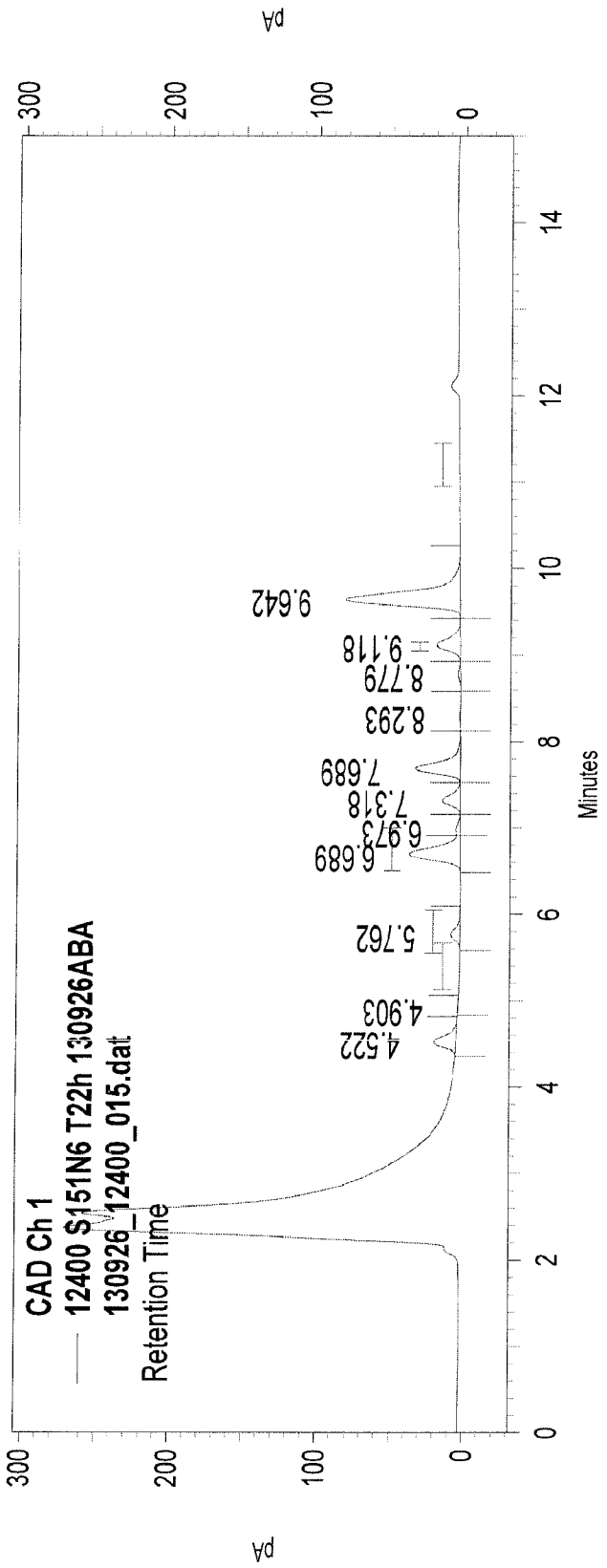

pA
300
200
100
0
CAD Ch 1
12400 S151N17 T5h 130927ABA
130927_12400_084.dat
Retention Time
5.491
7.010
7.295
7.677
8.728
9.630
11.227
0
2
4
6
8
10
12
14
Minutes

FIG. 65

FIG. 66


pA
300
200
100
0
CAD Ch 1
12400 S151N6 T22h 130926ABA
130926_12400_015.dat
Retention Time
4.522
4.903
5.762
6.689
6.973
7.318
7.689
8.293
8.779
9.118
9.642
0
2
4
6
8
10
12
14
Minutes

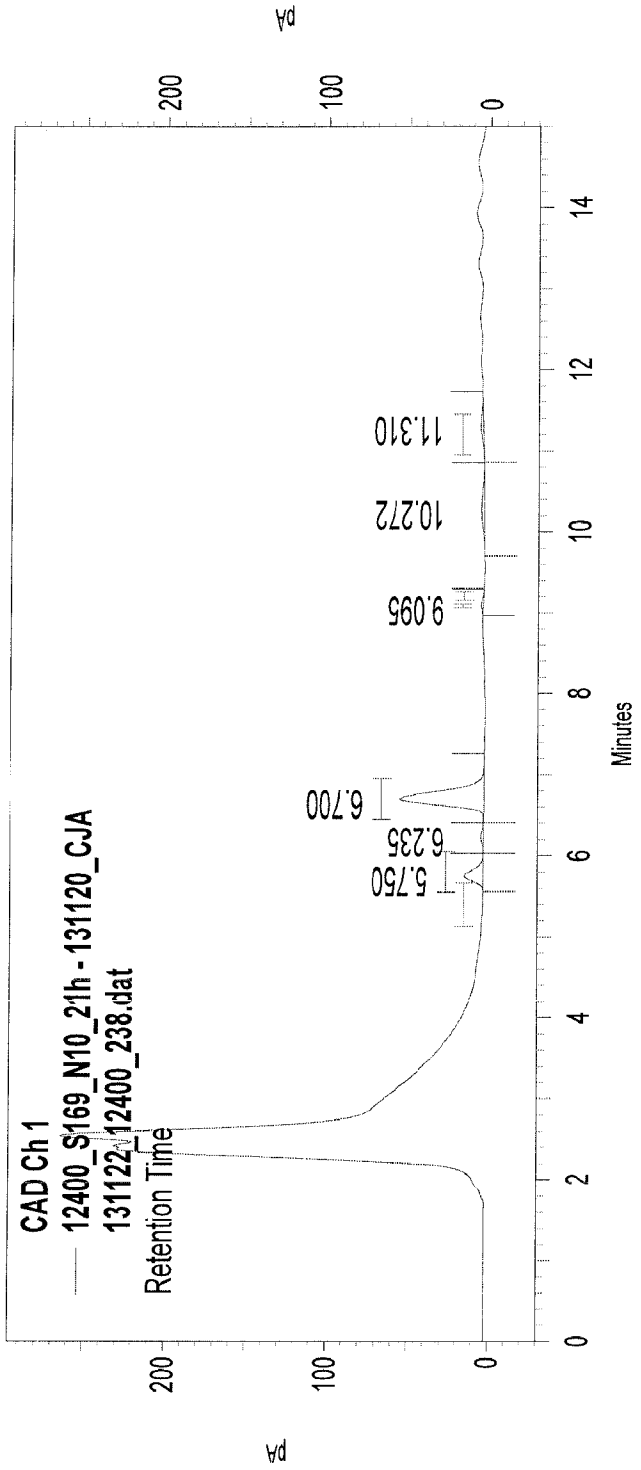


FIG. 67

Example 35

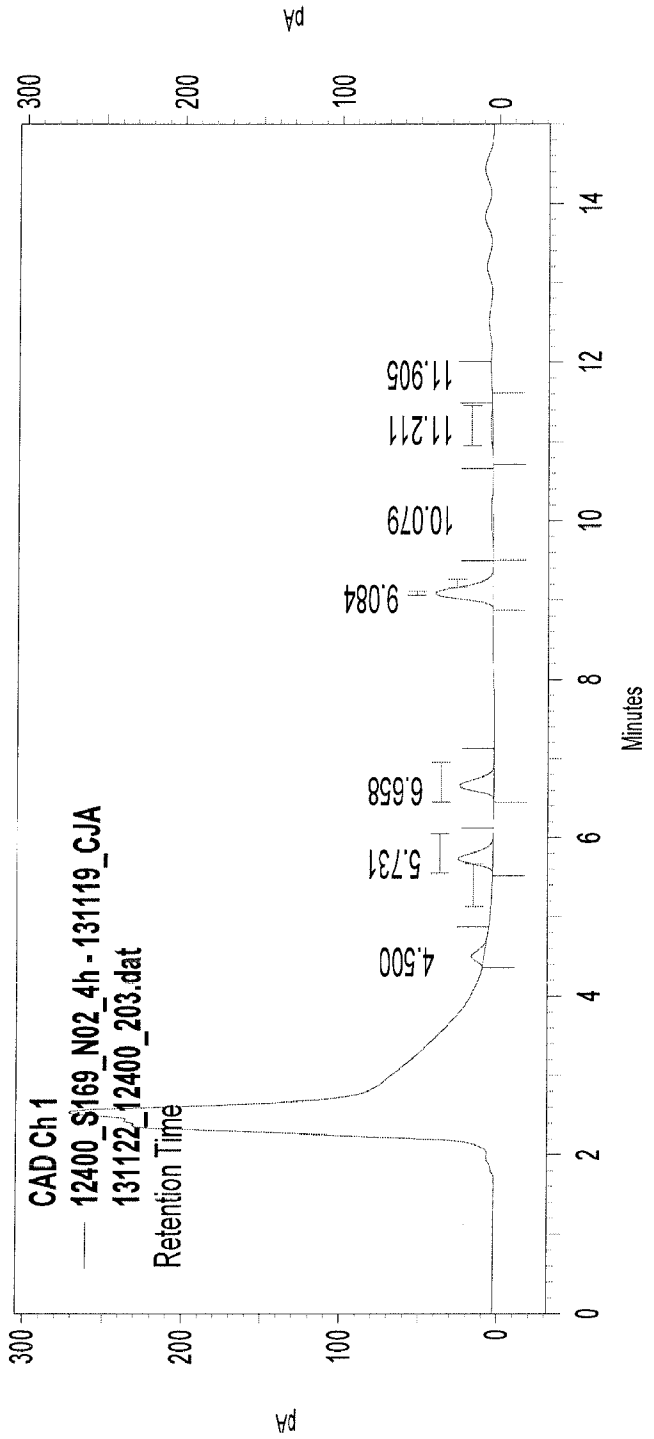


FIG. 68

Example 37

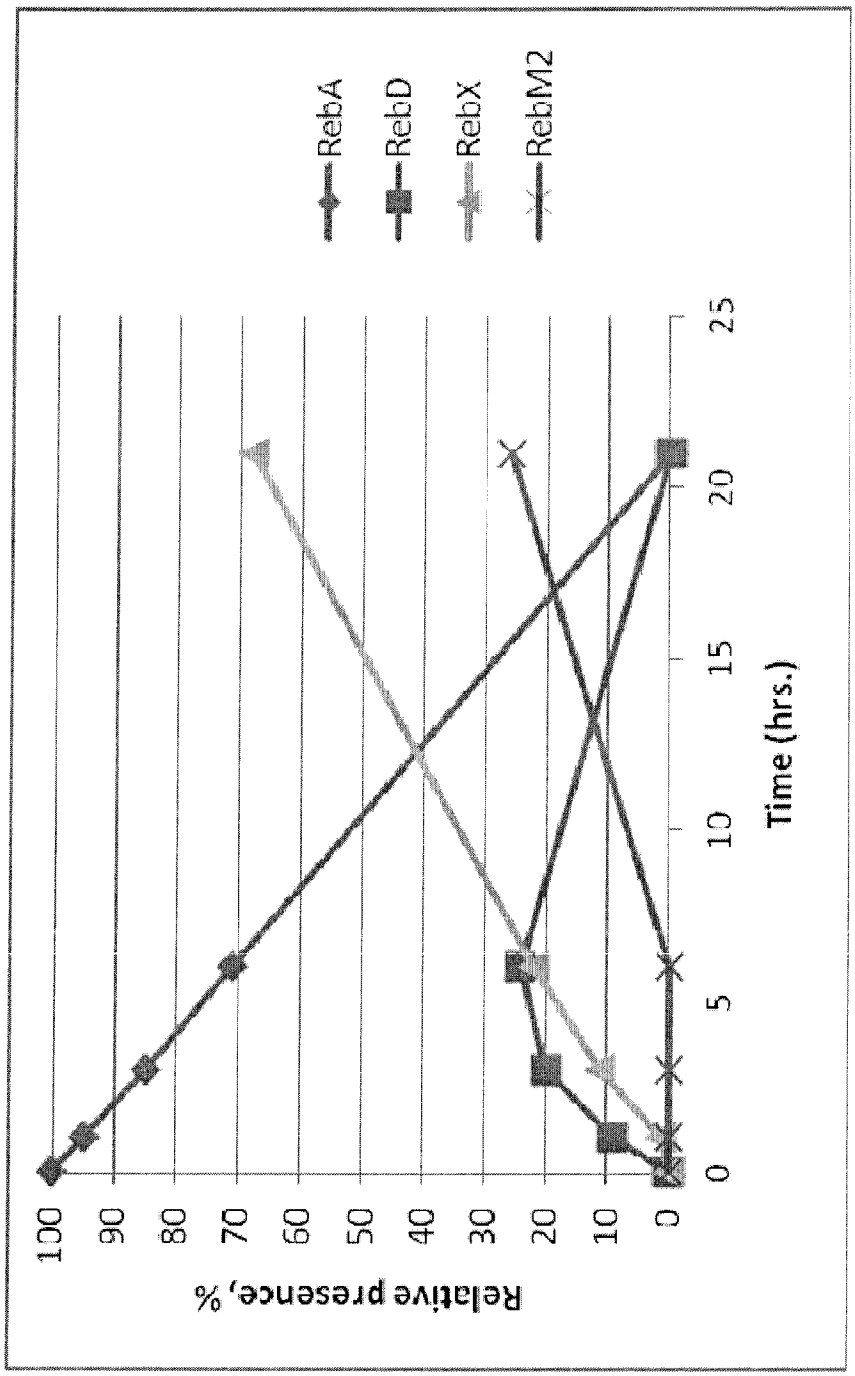


FIG. 69

Example 43

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US14/39666

A. CLASSIFICATION OF SUBJECT MATTER
IPC(8) - C07H 1/06; C12N, 9/10; C12P 19/56 (2014.01)
CPC - C12N 15/52, 15/8245; C07H 15/24
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC (8) - C07H 1/06, 15/24; C12N, 9/10, 15/52, 15/82; C12P 7/42, 19/56; C12Q 1/02 (2014.01); CPC - A23L 1/3002; C12N 9/1077, 15/52, 15/8243, 15/8245; C07H 15/24; C12P 7/42, 19/56; USPC - 426/658; 435/29, 78, 254.21, 419; 536/128

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

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
MicroPatent (US-G, US-A, EP-A, EP-B, WO, JP-bib, DE-C,B, DE-A, DE-T, DE-U, GB-A, FR-A); Google Scholar; IP.com; Proquest; steviol glycosides,rebaudioside M, Reb M, rebaudioside X, Rebaudioside M2, stevioside, rebaudioside D, biocatalyst, UDP-glycosyltransferases, UGT, UGT91D2, UGT76G1, UDP glucose recycling enzyme, sucrose synthase, steviol biosynthesis enzymes, sweetners, non-caloric, food, beverages, polyols, fermentation, microorganism, biocatalyst, biosynthesis, purity Eschierichia

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 2013/022989 A2 (HOUGHTON-LARSEN, J et al.) February 14, 2013; page 1, lines 9-14; page 11, lines 2-24; page 13, lines 18-25, page 20, lines 13-17; page 31, lines 16-21; page 32, lines 1-2; page 36, lines 26-30; page 37, lines 19-30; page 38, lines 2-3; page 45, line 10-15, page 95, lines 14-19; page 97, lines 1-27; page 98, lines 24-27; page 150, lines 1-14	1-2, 3/1-2, 4/1-2, 5-6, 7/1-2, 8/1-2, 9/1-2, 10/9/1-2, 12/1-2, 13/12/1-2, 16/1-2, 17/1-2, 42/1-2, 43/1-2
----		---
Y		11/9/1-2, 14/1-2, 15/14/1-2, 18/1-2
Y	US 8,030,481 B2 (PRAKASH, I et al.) October 4, 2011; column 3, lines 6-11; column 5, lines 25-28	14/1-2, 15/14/1-2
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☐ Further documents are listed in the continuation of Box C. ☐

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30 August 2014 (30.08.2014)	16 SEP 2014

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