Innovation, Sciences et Développement économique Canada
Office de la Propriété Intellectuelle du Canada

Innovation, Science and Economic Development Canada
Canadian Intellectual Property Office

CA 2963219 C 2022/11/15

(11)(21) 2 963 219

(12) BREVET CANADIEN
CANADIAN PATENT

(13) C

(86) Date de dépôt PCT/PCT Filing Date: 2014/11/03
(87) Date publication PCT/PCT Publication Date: 2015/05/07
(45) Date de délivrance/Issue Date: 2022/11/15
(85) Entrée phase nationale/National Entry: 2017/03/30
(86) N° demande PCT/PCT Application No.: US 2014/063682
(87) N° publication PCT/PCT Publication No.: 2015/066609
(30) Priorité/Priority: 2013/11/01 (US61/898,899)

(51) Cl.Int./Int.Cl. *A01H 9/00* (2006.01),
A01H 11/00 (2006.01), *C12N 15/52* (2006.01),
C12N 5/04 (2006.01)

(72) Inventeurs/Inventors:
BHUIYA, MOHAMMAD WADUD, US;
WANG, YECHUN, US;
YU, XIAODAN, US

(73) Propriétaire/Owner:
CONAGEN INC., US

(74) Agent: GOWLING WLG (CANADA) LLP

(54) Titre : PROCEDES D'UTILISATION D'O-METHYLTRANSFERASE POUR LA PRODUCTION BIOSYNTHETIQUE DE PTEROSTILBENE

(54) Title: METHODS OF USING O-METHYLTRANSFERASE FOR BIOSYNTHETIC PRODUCTION OF PTEROSTILBENE

(57) Abrégé/Abstract:
A biosynthetic method of making pterostilbene including expressing a 4- coumaratexoenzyme A ligase (4CL) in a cellular system, expressing a stilbene synthase (STS) in the cellular system, expressing a resveratrol O-methyltransferase (ROMT) in the cellular system, feeding p-coumaric acid to the cellular system, growing the cellular system in a medium, and producing pterostilbene.

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

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
7 May 2015 (07.05.2015)

WIPO | PCT

(10) International Publication Number
WO 2015/066609 A1

(51) **International Patent Classification**:
A01H 9/00 (2006.01) ***C12N 15/52*** (2006.01)
A01H 11/00 (2006.01) ***C12N 5/04*** (2006.01)

(21) **International Application Number**:
PCT/US2014/063682

(22) **International Filing Date**:
3 November 2014 (03.11.2014)

(25) **Filing Language**: English

(26) **Publication Language**: English

(30) **Priority Data**:
61/898,899 1 November 2013 (01.11.2013) US

(71) **Applicant**: **CONAGEN INC.** [US/US]; 15 DeAngelo Dr., Bedford, MA 01730 (US).

(72) **Inventors**: **BHUIYA, Mohammad, Wadud**; 1126 Bonhomme Lake Drive, Apt # A, Saint Louis, MO 63132 (US). **WANG, Yechun**; 7786 W Bruno Ave, Apt 3, Saint Louis, MO 63117 (US). **YU, Xiaodan**; 6231 Lexington Ridge Dr., Lexington, MA 02421 (US).

(74) **Agent**: **WU, George**; Husch Blackwell LLP, 190 Carondelet Plaza, Suite 600, St. Louis, MO 63105 (US).

(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, HN, HR, HU, ID, IL, IN, IR, IS, JP, KE, KG, KN, KP, KR, KZ, LA, LC, LK, LR, LS, 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, ST, 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 sequence listing part of description (Rule 5.2(a))*

WO 2015/066609 A1

(54) **Title**: METHODS OF USING O-METHYLTRANSFERASE FOR BIOSYNTHETIC PRODUCTION OF PTEROSTILBENE

(57) **Abstract**: A biosynthetic method of making pterostilbene including expressing a 4- coumaratexoenzyme A ligase (4CL) in a cellular system, expressing a stilbene synthase (STS) in the cellular system, expressing a resveratrol O-methyltransferase (ROMT) in the cellular system, feeding p-coumaric acid to the cellular system, growing the cellular system in a medium, and producing pterostilbene.

METHODS OF USING O-METHYLTRANSFERASE FOR BIOSYNTHETIC PRODUCTION OF PTEROSTILBENE

Technical Field

[0001] This disclosure has applicability in the food, medicinal, and pharmacological industries. This disclosure relates generally to methods for the biosynthetic production of pterostilbene utilizing O-methyltransferase (ROMT).

Background of the Disclosure

[0002] **Background Art:** Pterostilbene is a stilbenoid chemically related to resveratrol and is found in blueberries and grapes. It belongs to the group of phytoalexins, agents produced by plants to fight infections. Based on animal studies, it is thought to exhibit anti-cancer, anti-hypercholesterolemia, anti-hypertriglyceridemia properties, as well as the ability to fight off and reverse cognitive decline.

[0003] It is believed that the compound also has anti-diabetic properties, but so far very little has been studied on this issue.

[0004] Schmidlin et al. have reported that resveratrol O-methyltransferase (ROMT) could catalyze the direct conversion of resveratrol into pterostilbene (Schmidli et al, 2008). (Accession No: FM178870). Pterostilbene is produced by the action of 4-coumarate-CoA ligase (4CL), stilbene synthase (STS) and resveratrol O-methyltransferase (ROMT) (Figure 1).

Date Reçue/Date Received 2020-11-20

[0005] In this invention, Applicants demonstrate that ROMT can be expressed in a cellular system along with 4CL and STS to convert resveratrol into pterostilbene.

Brief Summary of Disclosure

[0006] The disclosure addresses the technical issue of producing pterostilbene in a cellular system, such as yeast or bacteria. Applicants have uniquely isolated the genes for 4-coumarate:coenzyme A ligase (4CL), stilbene synthase (STS), and resveratrol O-methyltransferase (ROMT) and expressed them in a cellular system that facilitate the production of pterostilbene. This disclosure provides for the industrial production of resveratrol and pterostilbene.

[0007] The present disclosure is a biosynthetic method of making pterostilbene comprising expressing a 4-coumarate:coenzyme A ligase (4CL) in a cellular system, expressing a stilbene synthase (STS) in the cellular system, expressing a resveratrol O-methyltransferase (ROMT) in the cellular system, feeding p-coumaric acid to the cellular system, growing the cellular system in a medium, and thereby, producing pterostilbene.

[0008] Another embodiment is a biosynthetic method of making pterostilbene comprising expressing a resveratrol O-methyltransferase (ROMT) in the cellular system, feeding resveratrol to the cellular system, growing the cellular system in a medium, and producing pterostilbene.

[0009] Another embodiment is a biosynthetic method of making resveratrol comprising expressing a 4-coumarate:coenzyme A ligase (4CL) in a cellular system, expressing a stilbene synthase (STS) in the cellular system, feeding p-coumaric acid to the cellular system, growing the cellular system in a medium, and producing resveratrol.

Date Reçue/Date Received 2020-11-20

[00010] Another embodiment is a biosynthetic method of making pterostilbene comprising expressing a 4-coumarate:coenzyme A ligase (4CL) in a first cellular system, expressing a stilbene synthase (STS) in the first cellular system, feeding p-coumaric acid to the first cellular system, growing the first cellular system in a medium, producing resveratrol, expressing a resveratrol O-methyltransferase (ROMT) in a second cellular system, feeding the produced resveratrol to the second cellular system, growing the second cellular system in a medium, and producing pterostilbene.

Brief Description of the Drawings

[00011] For a better understanding of the present disclosure, reference may be made to the accompanying drawings in which:

[00012] Figure 1 shows the biosynthetic pathway of pterostilbene.

[00013] Figure 2 shows HPLC profiles of three standards (p-coumaric acid, resveratrol and pterostilbene).

[00014] Figure 3 shows HPLC profiles of extracts from *E.coli* cells expressing 4CL::STS fusion gene.

[00015] Figure 4 shows HPLC profiles of extracts from *E.coli* cells expressing ROMT gene.

[00016] Figure 5 shows HPLC profiles of extracts from *E.coli* cells co-expressing 4CL::STS and ROMT gene.

Date Reçue/Date Received 2020-11-20

[00017] Figure 6 shows HPLC profiles of extracts from yeast cells expressing 4CL::STS fusion gene.

[00018] Figure 7 shows HPLC profiles of extracts from yeast cells expressing ROMT gene.

[00019] Figure 8 shows HPLC profiles of extracts from yeast cells co-expressing 4CL::STS and ROMT gene.

[00020] Figure 9 shows model of ROMT represented by ribbon. Substrates are represented by stick model in dark gray. Substrate binding residues are represented by stick model in black color. F167A, D174A, W258A, H261A (H261 is key amino acid) are changes made. They are all key amino acids for activity with H261 being the most important.

[00021] Figure 10 shows HPLC profiles of extracts from E.coli cells expressing wild-type ROMT and ROMT-mutant.

[00022] While the disclosure is susceptible to various modifications and alternative forms, specific embodiments thereof are shown by way of example in the drawing and will herein be described in detail. It should be understood, however, that the drawings and detailed description presented herein are not intended to limit the disclosure to the particular embodiment disclosed, but on the contrary, the intention is to cover all modifications, equivalents, and alternatives falling within the spirit and scope of the present disclosure as defined by the appended claims.

Date Reçue/Date Received 2020-11-20

Detailed Descriptions of the Disclosure

Definition

Cellular System

[00023] Cellular system is any cells that provide for the expression of ectopic proteins. It included bacteria, yeast, plant cells and animal cells. It includes both prokaryotic and eukaryotic cells. It also includes the in vitro expression of proteins based on cellular components, such as ribosomes.

Growing the Cellular System

[00024] Growing includes providing medium that would allow cells to multiply and divide. It also includes providing resources so that cells or cellular components can translate and make recombinant proteins.

Transfection

[00025] Transfection is the process of deliberately introducing nucleic acids into cells. The term is often used for non-viral methods in eukaryotic cells. It may also refer to other methods and cell types, although other terms are preferred: "transformation" is more often used to describe non-viral DNA transfer in bacteria, non-animal eukaryotic cells, including plant cells. In animal cells, transfection is the preferred term as transformation is also used to refer to progression to a cancerous state (carcinogenesis) in these cells. Transduction is often used to describe virus-mediated DNA transfer. Transformation, transduction, and viral infection are included under the definition of transfection for this application.

Date Reçue/Date Received 2020-11-20

Modified Amino Acid

[00026] A modified amino acid is one that has been chemically modified and it can be incorporated as part of a polypeptide sequence. The amino acid could be modified in a post-translational manner or prior to incorporation in the polypeptide sequence during translation.

4CL

[00027] The 4-coumarate:coenzyme A ligase is expressed from a *4CL* gene cloned from Arabidopsis thaliana (ecotype Columbia-0) (SEQ ID NO: 14). In another embodiment, the 4-coumarate coenzyme A ligase is expressed from a gene that has a sequence identity of at least 66% with a *4CL* gene cloned from Arabidopsis thaliana (ecotype Columbia-0) (SEQ ID NO: 14). In a further embodiment, the 4-coumarate:coenzyme A ligase is expressed from a gene that has a sequence similarity of at least 90% with a *4CL* gene cloned from Arabidopsis thaliana (ecotype Columbia-0) (SEQ ID NO: 14).

STS

[00028] The stilbene synthase is expressed from a *STS* gene cloned from grape (*Vitis vinifera*) (SEQ ID NO: 16). In another embodiment, the stilbene synthase is expressed from a gene that has a sequence identity of at least 66% with a *STS* gene cloned from grape (*Vitis vinifera*) (SEQ ID NO: 16). In a further embodiment, the stilbene synthase is expressed from a gene that has a sequence similarity of at least 90% with a *STS* gene cloned from grape (*Vitis vinifera*) (SEQ ID NO: 16).

ROMT

[00029] The resveratrol O-methyltransferase is expressed from a gene cloned from grape (*Vitis vinifera*) (SEQ ID NO: 12). In another embodiment, the resveratrol O-methyltransferase is expressed from a gene that has a sequence identity of at least 66% with a *ROMT* gene cloned from grape (*Vitis vinifera*) (SEQ ID NO: 12). In a further embodiment, the resveratrol O-methyltransferase is expressed from a gene that has a sequence similarity of at least 90% with a *ROMT* gene cloned from grape (*Vitis vinifera*) (SEQ ID NO: 12).

Date Reçue/Date Received 2020-11-20

[00030] An embodiment of the present disclosure is a biosynthetic method of making pterostilbene including expressing a 4-coumarate:coenzyme A ligase (4CL) in a cellular system, expressing a stilbene synthase (STS) in the cellular system, expressing a resveratrol O-methyltransferase (ROMT) in the cellular system, feeding p-coumaric acid to the cellular system, growing the cellular system in a medium, and producing pterostilbene.

[00031] In one embodiment, expressing the 4-coumarate:coenzyme A ligase and expressing the stilbene synthase comprise transfecting a *4CL::STS* fusion gene (SEQ ID NO: 18). In another embodiment, expressing the 4-coumarate:coenzyme A ligase comprises transfecting a *4CL* gene and expressing the stilbene synthase comprises transfecting a separate *STS* gene. Expressing the resveratrol O-methyltransferase comprises transfecting a *ROMT* gene.

[00032] The cellular system is selected from the group consisting of at least, bacteria, yeast, and a combination thereof. In another embodiment, the cellular system allows for ectopic biosynthetic reaction.

[00033] A further embodiment is a biosynthetic method of making pterostilbene comprising expressing a resveratrol O-methyltransferase (ROMT) in the cellular system, feeding resveratrol to the cellular system, growing the cellular system in a medium, and producing pterostilbene.

[00034] A further embodiment is a biosynthetic method of making resveratrol comprising expressing a 4-coumarate:coenzyme A ligase (4CL) in a cellular system, expressing a stilbene

Date Reçue/Date Received 2020-11-20

synthase (STS) in the cellular system, feeding p-coumaric acid to the cellular system, growing the cellular system in a medium, and producing resveratrol.

[00035] A further embodiment is a biosynthetic method of making pterostilbene comprising expressing a 4-coumarate:coenzyme A ligase (4CL) in a first cellular system, expressing a stilbene synthase (STS) in the first cellular system, feeding p-coumaric acid to the first cellular system, growing the first cellular system in a medium, producing resveratrol, expressing a resveratrol O-methyltransferase (ROMT) in a second cellular system, feeding resveratrol to the second cellular system, growing the second cellular system in a medium, and producing pterostilbene.

Materials and Methods

Strains, plasmids and culture condition

[00036] HI-Control 10G and DH5α were used for plasmid cloning, and BL21 (DE3) (Invitrogen) was used for recombinant protein expression in *E. coli*. Wat1 1 strain was used for protein expression in yeast. *p*-Coumaric acid, resveratrol and pterostilbene standard were all purchased from Sigma. The pETite N-His SUMO Kan Vector were purchased from Lucigen (Middleton, WI). Plasmid pETDuet-1 were purchased from Novagen was used recombinant protein expression purposes.

DNA manipulation

[00037] All DNA manipulations were performed according to standard procedures. Restriction enzymes and T4 DNA Ligase were purchased from New England Biolabs. All PCR amplification and cloning reactions were performed using Phusion® High-Fidelity DNA Polymerase New England Biolabs.

Date Reçue/Date Received 2020-11-20

RNA extraction and cDNA synthesis

[00038] ROMT (resveratrol O-methyltransferase) (SEQ ID NO: 13), 4CL (4-coumarate:coenzyme A ligase) (SEQ ID NO: 14)and STS (stilbene synthase) (SEQ ID NO: 17) were cloned from various plant species (specifically, from grape (Vitis vinifera) for the cloning of ROMT and STS and Arabidopsis thaliana (ecotype Columbia-0) for the cloning of 4CL). Plant total RNA was extracted from grape (*Vitis vinifera*) for the cloning of ROMT and STS and Arabidopsis thaliana (ecotype Columbia-0) for the cloning of 4CL with Trizol Plus RNA Purification Kit (Invitrogen Inc). The synthesis of cDNA was carried out with Im Prom-II™ Reverse Transcription System from Promega Inc. following the manufacturer's manual. The genes were amplified from the synthesized cDNA with New England Biolabs Phusion PCR Kit with the primers listed in Table 1.

Example 1

Construction of bacterial expression vector

[00039] The PCR product of ROMT was cloned into pETite N-His SUMO Kan Vector (Lucigen Inc) according to the manufacturer's manual. The resultant plasmid with the right insert was confirmed by sequencing, namely Sumo-ROMT, and was transformed into BL21(DE3) for heterogeneous gene expression.

[00040] To construct the 4CL::STS fusion gene (SEQ ID NO: 18), At4CL (SEQ ID NO: 14) and VvSTS (SEQ ID NO: 16) were fused using the PCR amplification strategy. The stop codon of 4CL was removed and a three amino acid linker (Gly-Ser-Gly) was introduced between the open reading frame of 4CL and STS. This construction resulted in a 2.87 kb fused gene construct encoding 4CL, the tripeptide linker, and STS. The fusion gene 4CL::STS cloned into the Gateway entry vector using the pCR8/GW/TOPO TA Cloning kit (Invitrogen), was transformed into One Shot E. coli cells, and then sequenced. 4CL::STS fusion gene was amplified and cloned into the multiple cloning site of pETDuet-1 vector via BamHI/HindIII, name pETDuet-4CLSTS. Primers for all cloning reactions are available in the Table 1.

Example 2

Construction of yeast expression vector

[00041] The 4CL::STS gene (SEQ ID NO: 18) was introduced into the *S. cerevisiae* Advanced Gateway destination vector pAG304GPD-ccdB (Addgene, Boston, MA), and the ROMT gene was swapped into another Gateway destination vector pAG305GPD-ccdB (Addgene) by LR clonase II enzyme mix kit (Invitrogen). The resultant plasmids were named pAG304GPD-4CLSTS and pAG304GPD-ROMT. The vectors contain integrative recombination side and an expression cassette under the control of a constitutive promoter (GPD). These vectors were transformed into WAT1 1 for fermentation assays.

Yeast transformation

[00042] The constructs, pAG304GPD-4CLSTS and pAG304GPD-ROMT, along with the pAG304GPD-ccdB and pAG305GPD-ccdB vectors as controls, were transformed into WAT11 cells with the Frozen-EZ Yeast Transformation II kit (Zymo Research, Orange, CA). Vectors, pAG304GPD-4CLSTS and pAG304GPD-ROMT, were co-tranformed into yeast WAT11 cells.

Homology modeling and docking for prediction of substrate binding residues of ROMT

[00043] According to applicants' knowledge, there is no tertiary structure of ROMT that can be used for analyses of substrate binding sites. To analyze the substrate binding site, applicants built a model for ROMT (Figure 9) with a computer program I-TASSER (Ambrish *et al.*, 2010). Applicants apply a combined method of molecular biology and structural biology for the laboratory evolution and development of enhanced ROMT. The substrate binding site was predicted by docking resveratrol with the ROMT model using the computer program SWISDOCK (Grosdidier *et al.*, 2011).

Date Reçue/Date Received 2020-11-20

The bioconversion of *p*-coumaric acid to resveratrol with the protein of 4CL::STS fusion protein in *E.coli* and *S. cerevisiae*

[00044] Single colony of the E coli strain was grown in 3 mL LB medium with 100 μg/mL ampicillin overnight at 37°C, and then the seed culture was transferred to 50 mL M9 modified medium with 100 μg mL ampicillin. E.coli BL21(DE3) containing pETDuet-4CLSTS vector was kept shaking at 200 rpm at 37°C in modified M9 medium until OD600 reach to 0.6, then added 1mM IPTG, after 2 hour induction with IPTG, *p*-coumaric acid was dissolved in 100% ethanol was added to the culture to 0.5 g/L. The culture was kept shaking under the same culture condition, and samples were taken at interval for HPLC analysis.

[00045] Wat11 cells containing pAG304GPD-4CLSTS plasmid were grown in SD drop out medium at 30°C until OD600 reach to 0.2, then add *p*-coumaric acid (0.5 g/L). The culture was kept shaking for 4 days under the same culture condition, and samples were taken at interval for HPLC analysis.

The bioconversion of resveratrol to pterostilbene with the protein of RQMT in *E.coli* and *S. cerevisiae*

[00046] *E.coli* BL21(DE3) containing SUMO-RMOT vector was grown in modified M9 medium at 37°C until OD600 reach to 0.6, then add 1mM IPTG, after 2 hour induction with IPTG, resveratrol dissolved in DMSO was added to the culture to 0.228 g /L. M9 medium was modified by addition of yeast extract (1.25g/L) and glycerol (0.5% v/v) into standard M9 medium. The culture was kept shaking under the same culture condition, and samples were taken at interval for HPLC analysis.

[00047] Wat11 cells containing pAG305GPD-RMOT plasmid were grown in standard yeast drop-out medium at 30°C until OD600 reach to 0.2, then add resveratrol acid (0.228 g/L).

Date Reçue/Date Received 2020-11-20

The culture was kept shaking under the same culture condition, and samples were taken at interval for HPLC analysis.

The bioconversion of *p*-coumaric acid to pterostilbene with the protein of ROMT and 4CL::STS fusion protein in *E.coli* and *S. cerevisiae*

[00048] *E.coli* BL21 (DE3) containing pETDuet-4CLSTS and SUMO-ROMT vectors was grown in modified M9 medium at 37°C until OD600 reach to 0.6, then add 1mM IPTG, after 2 hour induction with IPTG, *p*-coumaric acid dissolved in 100% ethanol was added to the culture to 0.5 g /L. The culture was kept shaking under the same culture condition, and samples were taken at interval for HPLC analysis.

[00049] Wat11 cells containing pAG304GPD-4CLSTS and pAG305GPD-ROMT plasmid were grown in SD drop out medium at 30°C until OD600 reach to 0.2, then add *p*-coumaric acid (0.5 g/L). The culture was kept shaking under the same culture condition, and samples were taken at interval for HPLC analysis.

Extraction of products

[00050] Aliquots of cultures (400ul) were extracted with 800ul of ethyl acetate. Extracts were evaporated to dryness with an Eppendorf Vacufuge (Eppendorf Scientific Westbury, NY) at room temperature and re-dissolved in 200ul of 80% (v/v) methanol.

HPLC analysis,

[00051] The HPLC analysis of resveratrol and pterostilbene was carried out with Dionex Ultimate 3000 system. Intermediates were separated by reverse-phase chromatography on a phenomenex Kinetex C18 column (particle size 2.6 μιη; 150 x 4.6 mm) with 0.1% (vol/vol) formic acid (Solution A) and 100% acetonitrile (Solution B). Samples were diluted into 80%

Date Reçue/Date Received 2020-11-20

methanol, and the following gradient procedure was used: 10% of solution B for 2 min; a linear gradient from 10% to 70% of solution B for 18 min; from 70% to 30% of solution B for 1 min; from 30% to 10% of solution B for 2 min; 10% of solution B for 5 min at a flow rate of 0.8ml/min. For quantification, all intermediates were calibrated with external standards. The compounds were identified by their retention times, as well as the corresponding spectra, which were identified with a diode array detector in the system.

Results

The byconversion of p-coumaric acid to resveratrol with fusion protein of 4CL and STS

[00052] Three standards were run by HPLC, which shows that they were separated well (Figure 2). With the PCR amplification strategy, 4CL and STS were fused with a link of Gly-Ser-Gly between 4CL and STS. Applicants tested the conversion of p-coumaric acid to resveratrol with the *E.coli* BL21(DE3) strain containing pETDuet-4CLSTS plasmid in modified M9 medium in the flasks. As shown in Figure 3, p-coumaric acid could be converted into resveratrol in modified M9 medium.

[00053] For in vivo yeast assay, fresh yeast colonies containing pAG304GPD-4CLSTS were grown at 30°C in 3ml yeast drop out medium containing 0.5g L p-coumaric acid for 4 days. Extracts were analyzed by HPLC. As shown in Figure 6, almost all *p*-coumaric acid was converted into resveratrol within 4 days. Compared with *E.coli*, the conversion efficiency in yeast was much better.

The bioconversion of resveratrol to pterostilbene with the protein of ROMT

[00054] As shown in Figure 4, resveratrol fed into the culture of *E.coli* with the expression of ROMT was converted into pterostilbene in the flask. HPLC analysis indicates resveratrol can be converted into pterostilbene in flask. However, there is another unknown peak, which

probably is that one of a methyl group added onto resveratrol. Similar results also were attained from yeast (Figure 7).

The bioconversion of p-coumaric acid to pterostilbene with co-expression 4CL::STS and ROMT

[00055] p-Coumaric acid was fed into the culture of *E.coli* and *S. cerevisiae* with the co-expression of 4CL::STS (SEQ ID NO: 19) and ROMT (SEQ ID NO: 13), as shown in Figure 5 and Figure 8, p-coumaric acid was converted into resveratrol and pterostilbene in the flask by HPLC with 24 h in *E.coli* and *S. cerevisiae*. Profiles of HPLC were obtained under the condition within 96 hours.

Conventional and Saturation mutagenesis of ROMT

[00056] After careful analysis of the substrate binding site, the amino acid residues 167, 174, 258, and 261 have been selected for saturation mutagenesis to improve the activity of ROMT (SEQ ID NO: 13). Applicants already performed conventional mutagenesis to construct F167A, D174A, W258A, and H261A mutants of ROMT to know their effect on enzyme activity. None of them show activity except D174A, which exhibited very low activity (Figure 10). This result suggests that the amino acid residues at sites 167, 174, 258 and 261 are important for substrate binding and catalytic activity. Therefore, next step applicants will perform site-directed saturation mutagenesis to improve the enzymatic activity of ROMT. Saturation mutagenesis allow change one amino acid to other alternative 19 amino acid residues. Applicants will perform saturation mutagenesis at the site 167, 174, 258, and 261 of ROMT by following the modified QuickChange site-directed mutagenesis strategy (Stratagene, CA) using NNK degenerate primers (N represents the mixture of A, T, G, C, and K for G/T). The codon NNK has 32-fold degeneracy and encodes all 20 amino acids without rare codons. The PCR mixture (25 µl) composed of Phusion HF buffer containing 60 ng Sumo-ROMT DNA template, 200 µM dNTPS, 0.5 µM forward primers, 0.5µM reverse primers, 5% DMSO and 0.3 µl polymerase. The PCR was performed by denaturing at 98°C for 20 sec, annealing at 58°C for 30 sec and followed by

elongation at 72°C for 2 min 30 sec for 25 cycle. The QuikChange PCR products were examined by agarose gel electrophoresis and then 15 µl of PCR products were digested with 1 µl Dpnl (New England Biolabs) at 37 °C for 4 hrs to remove the template plasmid. Aliquot of (2 µl) digestive products was added to 50 µl BL21(DE3) competent cells (Stratagene, CA), keep on ice for 30 min. After that, heat shock was done at 42°C for 20 sec, keep on ice for 2 min and then 500 µl SOC medium was added and grow the cells at 37 °C for 1 hr. The cells were centrifuged at 5000 rpm for min, 450 µl supernatant was discarded and cells were suspended with the rest of the SOC medium and were inoculated on Luria-Bertani (LB) agar plates containing kanamycin (50 µg/ml). We will isolate the plasmid and DNA sequencing to confirm the mutant. We will confirm the quality of the library by DNA sequencing.

Table 1. Primers used in this study

[00057]

Name	Sequence (5 '-3')
SumoROMTF	CGC GAA CAG ATT GGA GGT GAT TTG GCA AAC GGT GTG ATA TCA GC (SEQ ID NO: 1)
SumoROMTR	GTG GCG GCC GCT CTA TTA TCA AGG ATA AAC CTC AAT GAG GGA CC (SEQ ID NO: 2)
ROMTF	ATG GAT TTG GCA AAC GGT GTG ATA TC (SEQ ID NO: 3)
ROMTR	TCA AGG ATA AAC CTC AAT GAG GGA CC (SEQ ID NO: 4)
4CL-F	ATG GCG CCA CAA GAA CAA GCA GTT TC (SEQ ID NO: 5)
4CLSTS-LinkF	GAG GGC AAA ACT AGC AAA TGG ATT GGG ATC TGG CAT GGC TTC AGT CGA GGA ATT TAG AA(SEQ ID NO: 6)
4CLSTS-LinkR	TTC TAA ATT CCT CGA CTG AAG CCA TGC CAG ATC CCA ATC CAT TTG CTA GTT TTG CCC TC(SEQ ID NO: 7)
STS-R	TTA ATT TGT AAC CAT AGG AAT GCT ATG (SEQ ID NO: 8)
4CL-BamHIF	CGG GAT CCA TGG CGC CAC AAG AAC AAG CAG TTT C (SEQ ID NO: 9)
STS-HindlllR	CCC AAG CTT TTA ATT TGT AAC CAT AGG AAT GCT ATG (SEQ ID NO: 10)
Oligo dT (22)	TTT TTT TTT TTT TTT TTT TTV N (SEQ ID NO: 11)

Identity and similarity

[00058] Identity is the fraction of amino acids that are the same between a pair of sequences after an alignment of the sequences (which can be done using only sequence information or structural information or some other information, but usually it is based on sequence information alone), and similarity is the score assigned based on an alignment using

some similarity matrix. The similarity index can be any one of the following BLOSUM62, PAM250, or GONNET, or any matrix used by one skilled in the art for the sequence alignment of proteins.

[00059] Identity is the degree of correspondence between two sub-sequences (no gaps between the sequences). An identity of 25% or higher implies similarity of function, while 18-25% implies similarity of structure or function. Keep in mind that two completely unrelated or random sequences (that are greater than 100 residues) can have higher than 20% identity. Similarity is the degree of resemblance between two sequences when they are compared. This is dependent on their identity.

[00060] As is evident from the foregoing description, certain aspects of the present disclosure are not limited by the particular details of the examples illustrated herein, and it is therefore contemplated that other modifications and applications, or equivalents thereof, will occur to those skilled in the art. It is accordingly intended that the claims shall cover all such modifications and applications that do not depart from the spirit and scope of the present disclosure.

[00061] Moreover, unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by one of ordinary skill in the art to which the disclosure belongs. Although any methods and materials similar to or equivalent to or those described herein can be used in the practice or testing of the present disclosure, the preferred methods and materials are described above.

[00062] Other aspects, objects and advantages of the present disclosure can be obtained from a study of the drawings, the disclosure and the appended claims.

Date Reçue/Date Received 2020-11-20

References

Schmidlin L, Poutaraud A, Claudel P, Mestre P, Prado E, Santos-Rosa M, Wiedemann-Merdinoglu S, Karst F, Merdinoglu D, Hugueney P (2008) A stress-inducible resveratrol O-methyltransferase involved in the biosynthesis of pterostilbene in grapevine. Plant Physiol. l48(3): 1630-1639.

Ambrish R, Alper K, Yang Z (2010) I-TASSER: a unified platform for automated protein structure and function prediction. *Nature Protocols*, 5:725-738.

Grosdidier A, Zoete V, Michielin O. (2011) SwissDock, a protein-small molecule docking web service based on EADock DSS.JNucleic Acids Res. 39:W270-277.

Date Reçue/Date Received 2020-11-20

CLAIMS

What is claimed is:

1. A biosynthetic method of making pterostilbene comprising:

expressing a 4-coumarate:coenzyme A ligase (4CL) in a cellular system;

expressing a stilbene synthase (STS) in the cellular system;

expressing a resveratrol O-methyltransferase (ROMT) in the cellular system;

feeding p-coumaric acid to the cellular system;

growing the cellular system in a medium; and

producing pterostilbene,

wherein the 4-coumarate:coenzyme A ligase is expressed from a gene that has a sequence identity of at least 66% with a 4CL gene comprising the sequence of SEQ ID NO: 14, wherein the resulting 4-coumarate:coenzyme A ligase has at least the same or similar ligase activity as the 4CL expressed from the gene consisting of SEQ. ID. NO. 14;

wherein the stilbene synthase is expressed from a gene that has a sequence identity of at least 66% with a STS gene comprising the sequence of SEQ ID NO: 16, where the resulting stilbene synthase has at least the same or similar synthase activity as the STS expressed from the gene consisting of SEQ. ID. NO. 16;

wherein the resveratrol O-methyltransferase is expressed from a gene that has a sequence identity of at least 66% with a ROMT gene comprising the sequence of SEQ ID NO: 12, and wherein the resveratrol O-methyltransferase expressed is modified at one or more residues in SEQ ID NO: 13 selected from the list consisting of residues 167, 174, 258, 261, and a combination thereof, by an alternative amino acid or a modified amino acid, and wherein the modified resveratrol O-methyltransferase exhibits increased activity in converting resveratrol to pterostilbene relative to an unmodified resveratrol O-methyltransferase comprising the sequence of SEQ ID NO: 13.

2. The biosynthetic method of making pterostilbene of claim 1, wherein *4CL* gene is cloned from *Arabidopsis thaliana*.

Date reçue / Date received 2021-11-04

3. The biosynthetic method of making pterostilbene of claim 1, wherein the 4-coumarate:coenzyme A ligase is expressed from a gene that has a sequence identity of at least 90% with a 4CL gene comprising the sequence of SEQ ID NO: 14.

4. The biosynthetic method of making pterostilbene of claim 1, wherein the stilbene synthase is expressed from a STS gene cloned from grape.

5. The biosynthetic method of making pterostilbene of claim 1, wherein the stilbene synthase is expressed from a gene that has a sequence identity of at least 90% with a STS gene comprising the sequence of SEQ ID NO: 16.

6. The biosynthetic method of making pterostilbene of claim 1, wherein the resveratrol O-methyltransferase is expressed from a gene that has a sequence identity of at least 90% with a ROMT gene comprising the sequence of SEQ ID NO. 12.

7. The biosynthetic method of making pterostilbene of claim 1, wherein expressing the 4-coumarate:coenzyme A ligase and expressing the stilbene synthase comprise transfecting the 4CL gene and the STS gene as a 4CL::STS fusion gene.

8. The biosynthetic method of making pterostilbene of claim 1, wherein expressing the 4-coumarate:coenzyme A ligase comprises transfecting the 4CL gene.

9. The biosynthetic method of making pterostilbene of claim 1, wherein expressing the stilbene synthase comprises transfecting the STS gene.

10. The biosynthetic method of making pterostilbene of claim 1, wherein expressing the resveratrol O-methyltransferase comprises transfecting the ROMT gene.

11. The biosynthetic method of making pterostilbene of claim 1, wherein the cellular system is selected from the group consisting of bacteria, yeast, plant cells, animal cells and a combination thereof.

Date reçue / Date received 2021-11-04

12. The biosynthetic method of making pterostilbene of claim 1, wherein the cellular system allows for ectopic biosynthetic reaction.

13. The biosynthetic method of making pterostilbene of claim 1, wherein the cellular system comprises an in vitro translation system.

14. A biosynthetic method of making pterostilbene comprising:

expressing a resveratrol O-methyltransferase (ROMT) in a cellular system;

feeding resveratrol to the cellular system;

growing the cellular system in a medium; and

producing pterostilbene,

wherein, the resveratrol O-methyltransferase expressed is modified at one or more of its residues in SEQ I D NO: 13 selected from the list consisting of residues 167, 174, 258, 261, and a combination thereof, by an alternative amino acid or a modified amino acid, and wherein the modified resveratrol O-methyltransferase exhibits increased activity in converting resveratrol to pterostilbene relative to an unmodified resveratrol O-methyltransferase comprising the sequence of SEQ ID NO: 13.

15. A biosynthetic method of making resveratrol comprising:

expressing a 4-coumarate:coenzyme A ligase (4CL) in a cellular system;

expressing a stilbene synthase (STS) in the cellular system;

expressing a resveratrol O-methyltransferase (ROMT) in the cellular system;

feeding p-coumaric acid to the cellular system;

growing the cellular system in a medium; and

producing resveratrol,

wherein, the resveratrol O-methyltransferase expressed is modified at one or more of its residues in SEQ I D NO: 13 selected from the list consisting of residues 167, 174, 258, 261, and a combination thereof, by an alternative amino acid or a modified amino acid, and wherein the modified resveratrol O-methyltransferase exhibits increased activity in converting resveratrol to pterostilbene relative to an unmodified resveratrol O-methyltransferase comprising the sequence of SEQ ID NO: 13.

Date reçue / Date received 2021-11-04

16. A biosynthetic method of making pterostilbene comprising:

expressing a 4-coumarate:coenzyme A ligase (4CL) in a first cellular system;

expressing a stilbene synthase (STS) in the first cellular system;

feeding p-coumaric acid to the first cellular system;

growing the first cellular system in a medium;

producing resveratrol;

expressing a resveratrol O-methyltransferase (ROMT) in a second cellular system;

feeding resveratrol produced in the first cellular system to the second cellular system;

growing the second cellular system in a medium; and

producing pterostilbene,

wherein, the resveratrol O-methyltransferase expressed is modified at one or more of its residues in SEQ ID NO: 13 selected from the list consisting of residues 167, 174, 258, 261, and a combination thereof, by an alternative amino acid or a modified amino acid, and wherein the modified resveratrol O-methyltransferase exhibits increased activity in converting resveratrol to pterostilbene relative to an unmodified resveratrol O-methyltransferase comprising the sequence of SEQ ID NO: 13.

Date reçue / Date received 2021-11-04

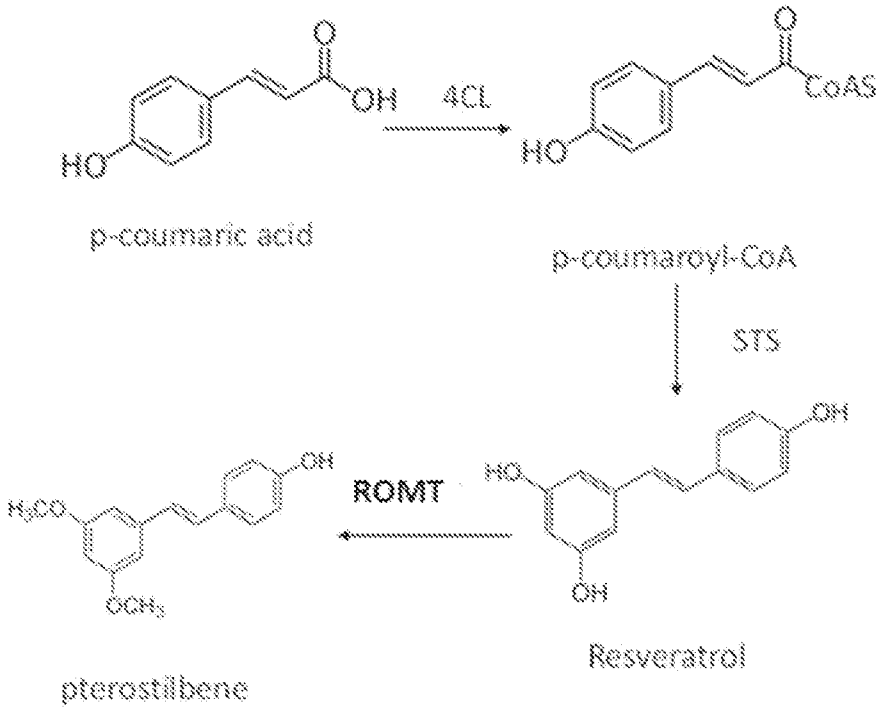
HO
OH
4CL
HO
CoAS
p-coumaric acid
p-coumaroyl-CoA
STS
OH
HO
OH
ROMT
H3CO
OCH3
OH
pterostilbene
Resveratrol

FIG. 1

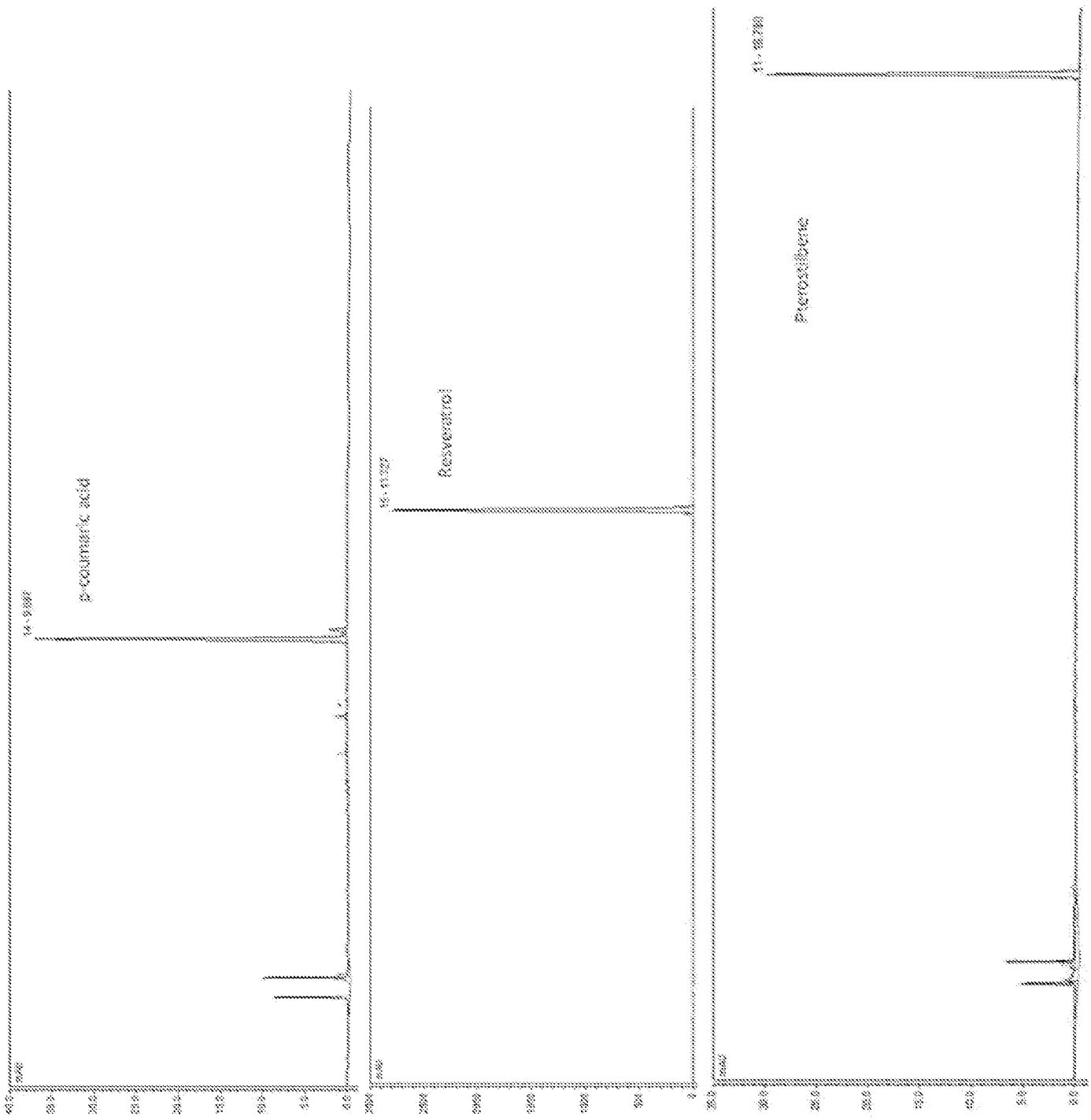

FIG. 2

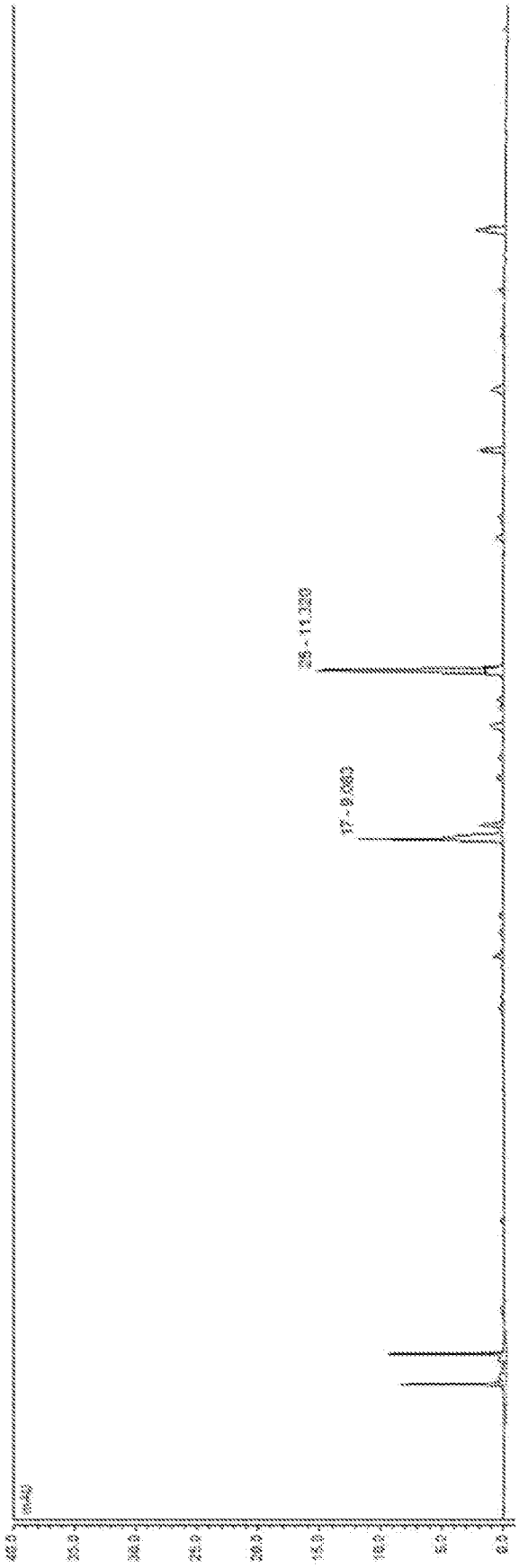

FIG. 3

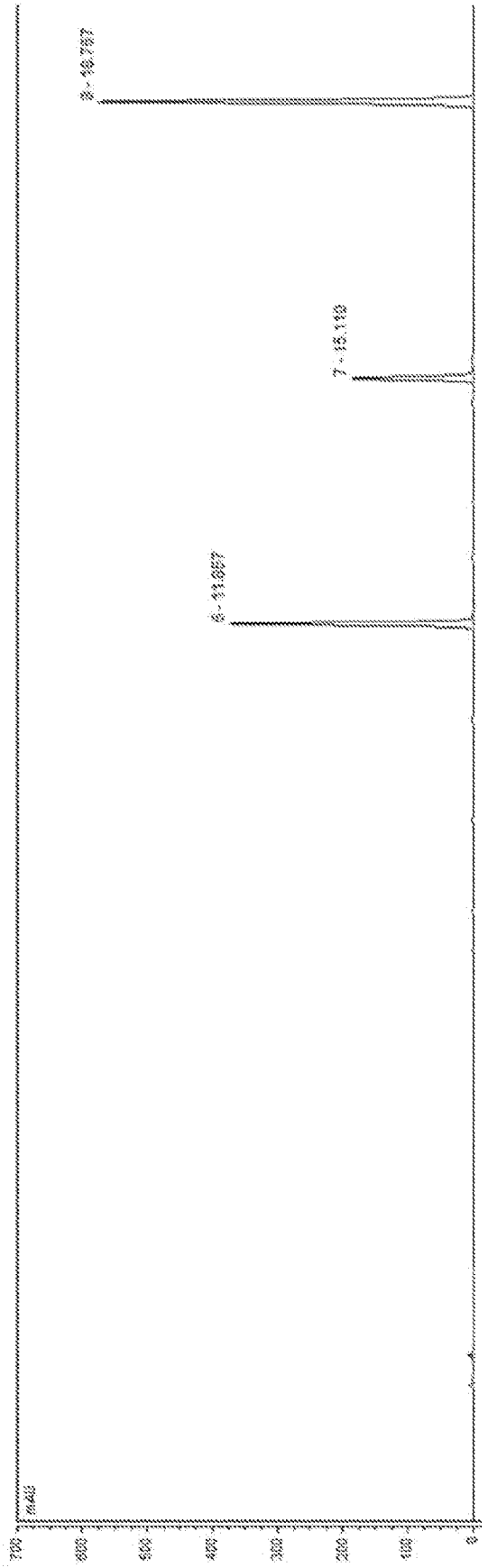

FIG. 4

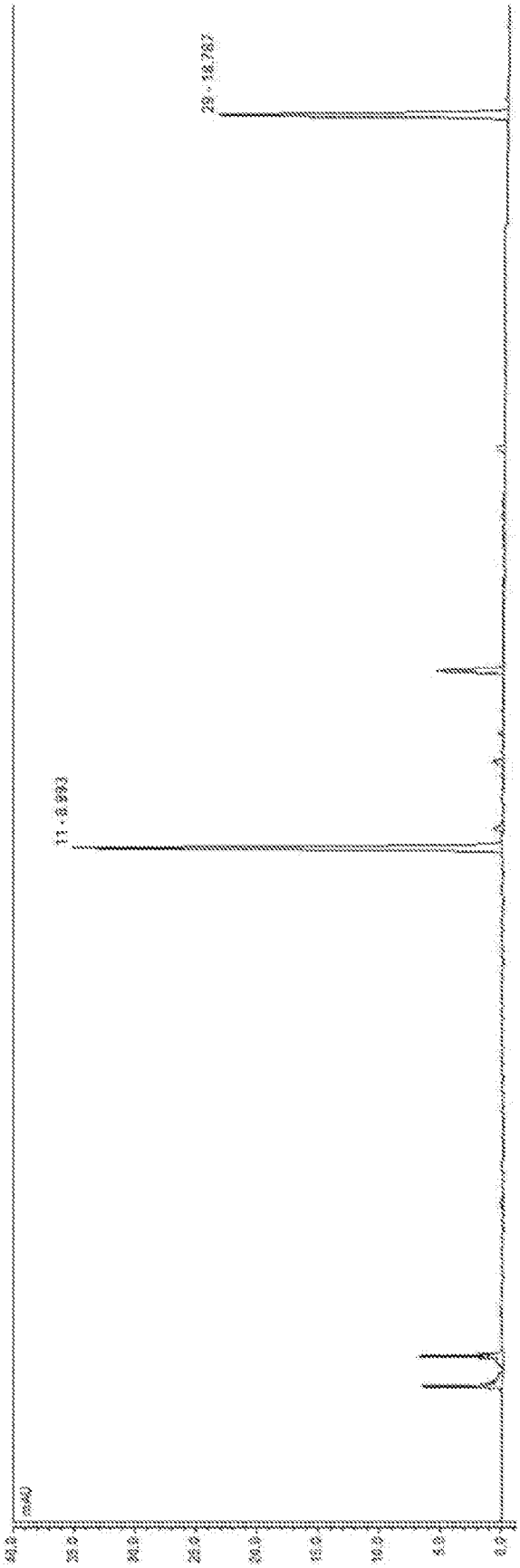

FIG. 5

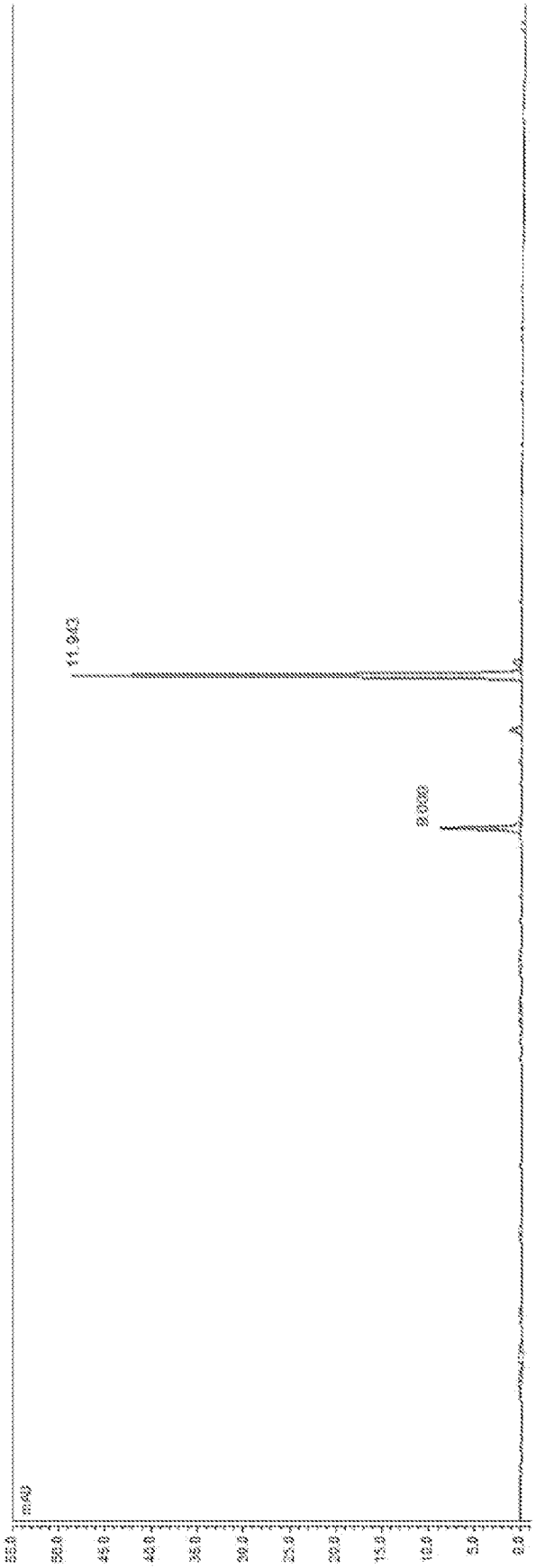

FIG. 6

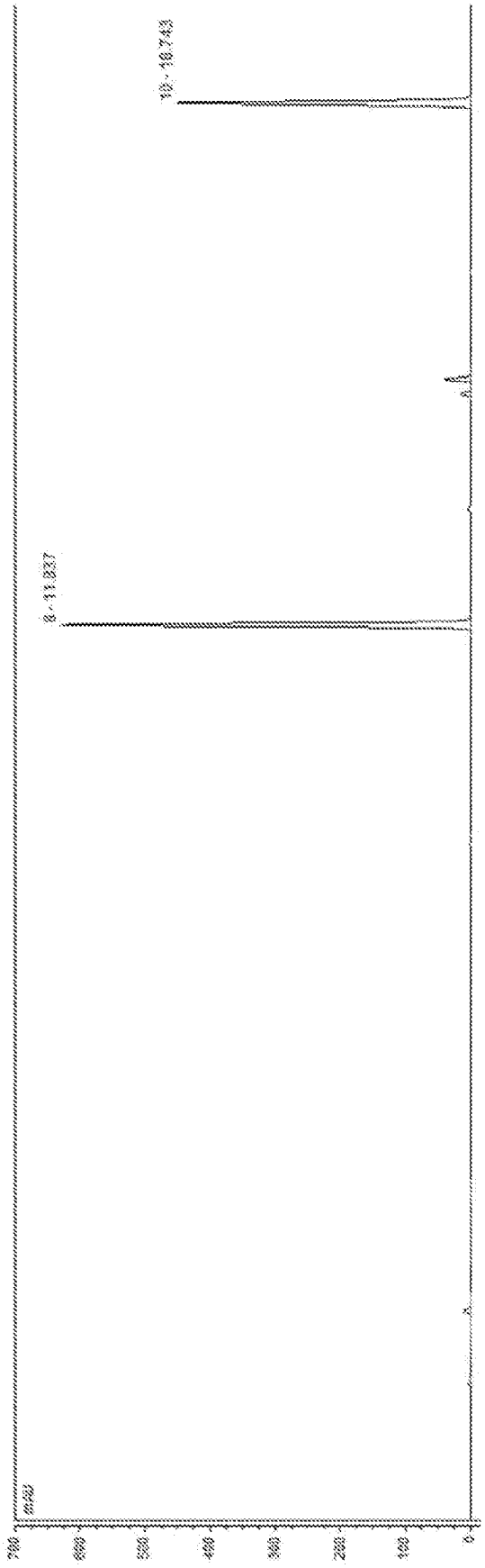

FIG. 7

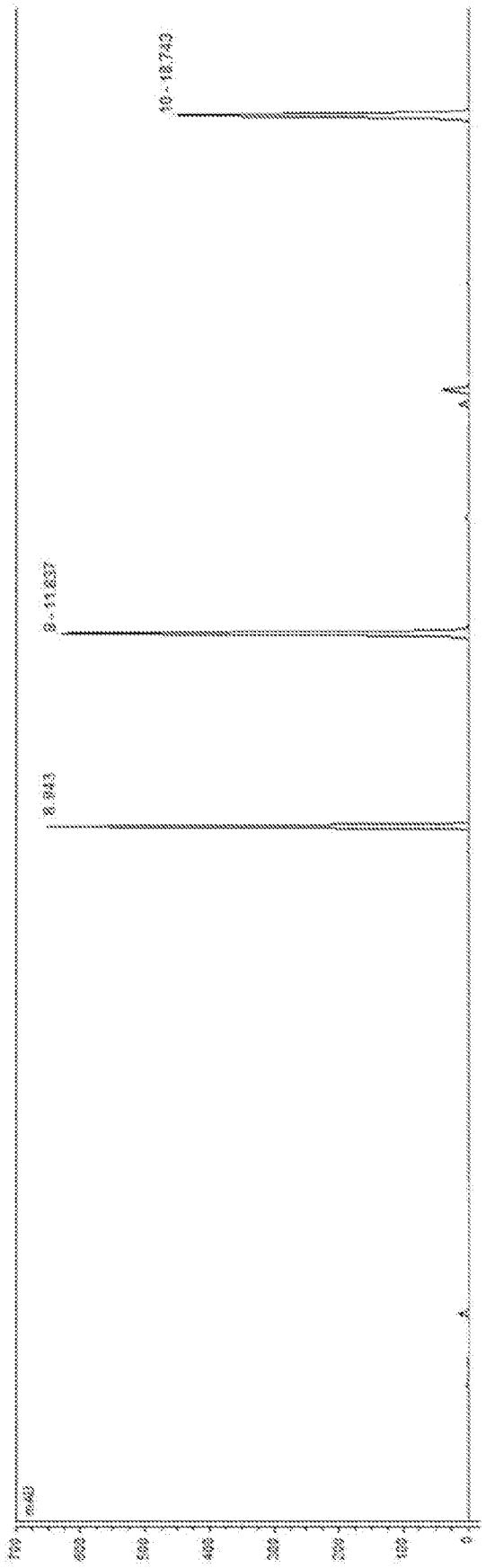

FIG. 8

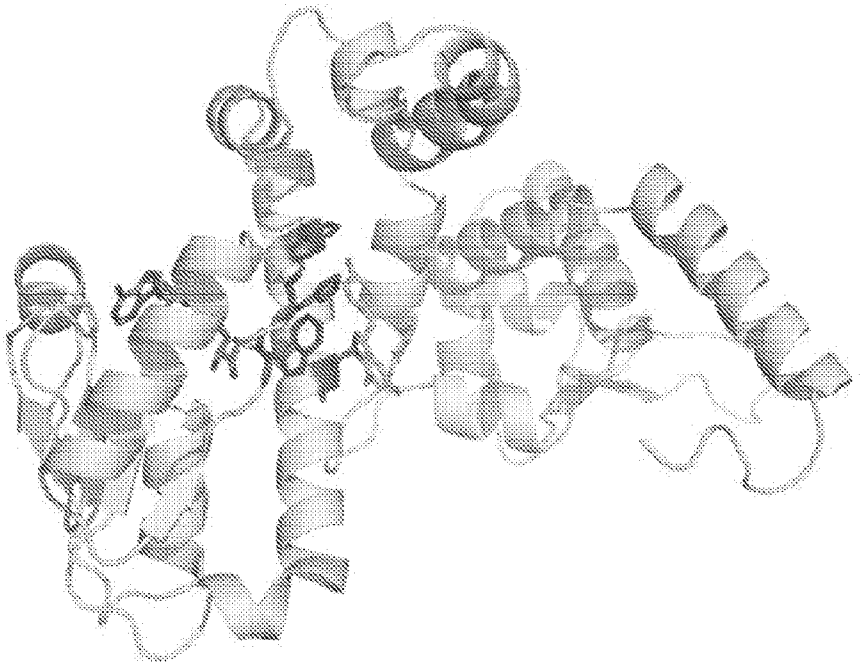

FIG. 9

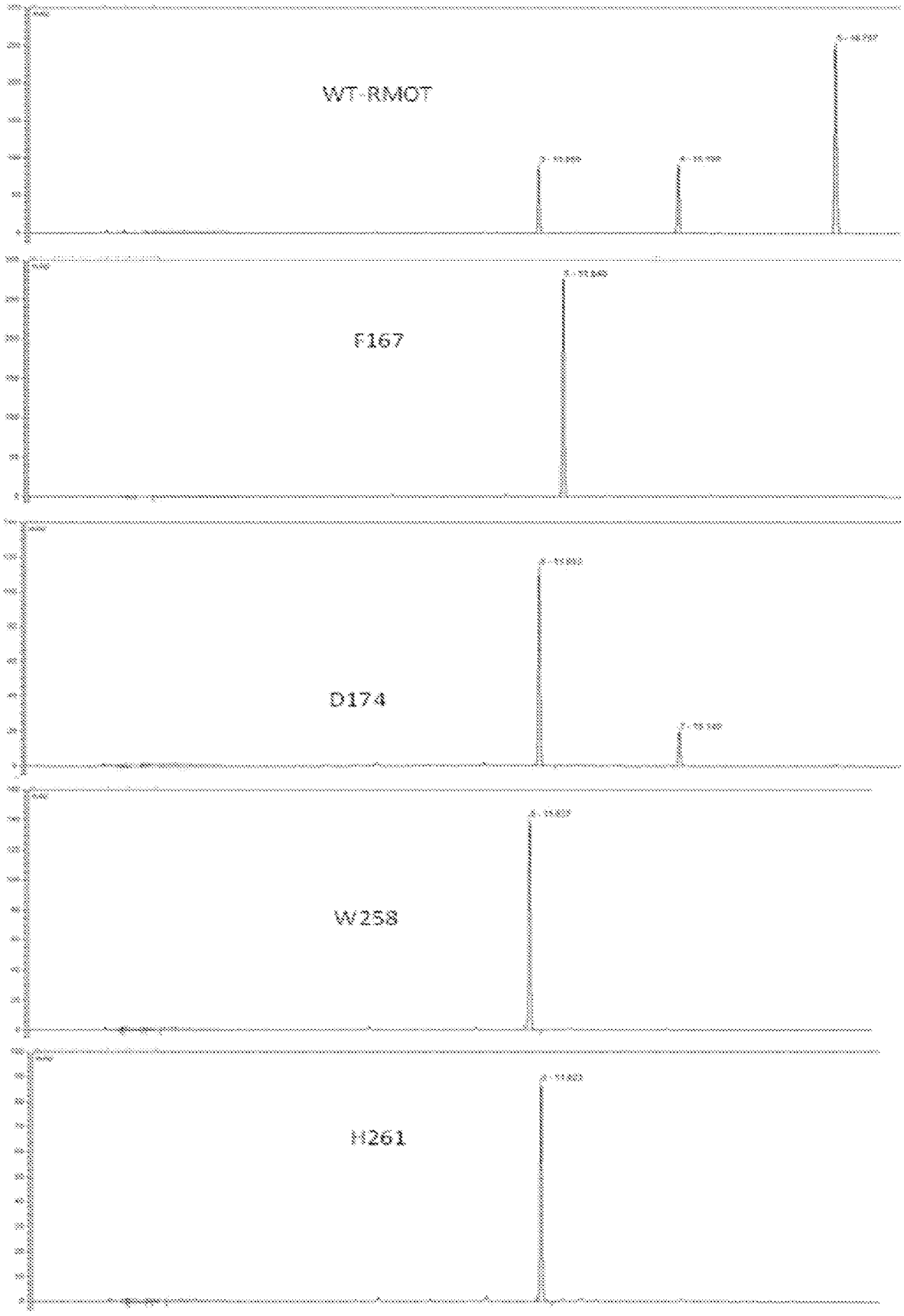

FIG. 10