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(54) Title: CONSTRUCTS AND METHODS FOR BIOSYNTHESIS OF CYCLOPAMINE

(57) Abstract: The present disclosure relates generally to the identification of enzymes within the cyclopamine biosynthesis pathway as well as to engineering transgenic plants or organisms for the production of cyclopamine.

CONSTRUCTS AND METHODS FOR BIOSYNTHESIS OF CYCLOPAMINE

CROSS REFERENCE TO RELATED APPLICATIONS

[0001] The present application claims priority to U.S. Provisional Application No. 62/018,556, filed June 28, 2014, entitled "Constructs and Methods for Biosynthesis of Cyclopamine," and U.S. Provisional Application No. 62/152,489 filed April 24, 2015 entitled "Constructs and Methods for Biosynthesis of Cyclopamine," both of which are herein incorporated by reference.

STATEMENT REGARDING FEDERALLY SPONSORED RESEARCH

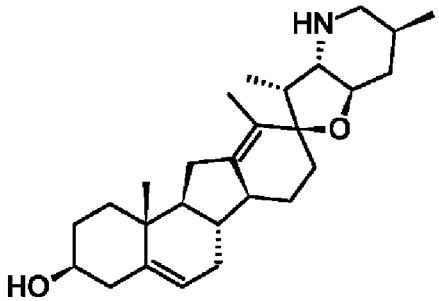
[0002] This invention was funded in part by Grant No. 1RO1DAO2517-02 from the National Institutes of Health. The government has certain rights in the invention.

INCORPORATION BY REFERENCE OF THE SEQUENCE LISTING

[0003] The accompanying "Sequence Listing" forms a part of this application and the sequences disclosed therein are herein incorporated by reference.

BACKGROUND

[0004] Cyclopamine (11-deoxojervine):



is a naturally occurring alkaloid isolated from the corn lily (*Veratrum californicum*). It belongs to the group of steroidal jerveratrum alkaloids, and causes usually fatal birth defects such as preventing the fetal brain from dividing into two lobes (holoprosencephaly) and inducing cyclopian teratogenic effects in sheep, causing the development of a single eye (cyclopia). It does so by inhibiting the hedgehog signaling pathway (Hh), and is therefore useful in studying the role of Hh in normal development.

[0005] Inappropriate activation of the Hh can also trigger cancer in adult humans, leading to basal cell carcinoma, medulloblastoma, rhabdomyosarcoma, and prostate, pancreatic, and breast cancers. Cyclopamine appears to act as a primary inhibitor of the Hh in cells, and

controlling the pathway using cyclopamine could provide a way to treat cancers in which the Hh is overexpressed. It is currently being investigated as a treatment agent in basal cell carcinoma, medulloblastoma, and rhabdomyosarcoma, which are tumors that result from excessive Hh activity, glioblastoma, and as a treatment agent for multiple myeloma.

[0006] Cyclopamine has thus far been described from the California corn lily *Veratrum californicum* and the Japanese species *Veratrum glandiflorum*. Cyclopamine is of pharmacological interest as a Hedgehog signaling pathway inhibitor. Cyclopamine was discovered to inhibit the Hedgehog signaling pathway by direct binding to the G protein-coupled receptor Smoothened. As such, it has shown promising antineoplastic activities against several cancers in which Hedgehog signaling pathway malfunction is implicated, including pancreatic cancer, renal cell carcinoma, medulloblastoma, and leukemia. A semi-synthetic analog of cyclopamine, IPI-926, has been in clinical trials for treatment of several cancers including metastatic solid tumors, pancreatic cancer and leukemia. Due to a complicated total synthesis, wild-collected *V. californicum* is the current source of cyclopamine. Cultivation of the plant has not been achieved. Coupled with slow growth in the wild, this makes cyclopamine an attractive target for biotechnological production.

[0007] Little is known about the cyclopamine biosynthetic pathway. Studies performed in the 1960's and 1970's on *V. californicum* and *V. grandiflorum* provide a general framework for the pathway and described hypothetical precursors, intermediates, and related compounds found *in planta*. Cholesterol has been shown to be a common precursor to the steroid alkaloids in this and similar pathways. Thus far, none of the genes involved in cyclopamine biosynthesis has been identified.

[0008] In view of the potential importance of cyclopamine and verazine metabolite derivatives with improved properties as a cancer therapeutics, the need for adequate supplies of these compounds to facilitate development of these molecules for patients, the complex chemical synthesis of cyclopamine, and the supply constraints imposed by wild collection of the source plant *Veratrum californicum*, there is a need in the art for methods to enhance the accumulation of this compound in plants via the development of a synthetic biology production platform. Engineering of the cyclopamine biosynthetic pathway into an easily cultivated host plant can result in an economically attractive, sustainable supply of this drug to meet future market demand. However, improved *in planta* production requires knowledge of the underlying biosynthetic genes, which is currently lacking.

SUMMARY

[0009] Accordingly, to address this need, disclosed herein is a broadly applicable biosynthetic gene discovery method based on correlating cyclopamine accumulation with RNAseq gene expression data.

[0010] Further scope of the applicability of the presently disclosed embodiments will become apparent from the detailed description and drawings provided below. However, it should be understood that the detailed description and specific examples, while indicating preferred embodiments of this disclosure, are given by way of illustration only since various changes and modifications within the spirit and scope of these embodiments will become apparent to those skilled in the art from this detailed description.

BRIEF DESCRIPTION OF THE DRAWINGS

[0011] The foregoing and other aspects, features, and advantages of the present disclosure will be better understood from the following detailed description taken in conjunction with the accompanying figures, all of which are given by way of illustration only, and are not limitative of the present specification, in which:

[0012] Figure 1. Enzyme assay workflow for clarification of cyclopamine biosynthetic pathway. Assays were performed with crude *S. frugiperda* Sf9 cells infected with baculovirus containing select *V. californicum* genes. Each arrow represents an extraction step; the resulting product was utilized as substrate for the subsequent enzyme assay. All cytochromes P450 were co-expressed with *E. californica* cytochrome P450 reductase (CPR). (A) 12 assays each of 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxy-26-aminocholesterol 22-oxidase, and control cytochrome P450 each with pure 22(*R*)-hydroxycholesterol were incubated and extracted. Dried extracts from each were split according to panel A. Final extracts were analyzed by gas chromatography mass spectrometry with results shown in Figure 16. (B) 8 assays of cholesterol 22-hydroxylase and 22-hydroxycholesterol 26-hydroxylase/oxidase and 8 assays of cholesterol 22-hydroxylase and 22-hydroxy-26-aminocholesterol 22-oxidase were incubated and extracted. Dried extracts were split and used as substrate in 2 more enzyme assays, 4 reactions each. These were extracted then split into 2 more assays, 2 reactions each. Products at each step were analyzed by liquid chromatography mass spectrometry with results shown in Figure 11.

[0013] Figure 2. Cyclopamine accumulation profile in *V. californicum*. Each *V. californicum* tissue underwent three independent ethanol extractions followed by liquid chromatography mass spectrometry analysis on a 4000 QTRAP. Quantitation with authentic cyclopamine was accomplished using a standard curve with peak areas. Each value represents ng of alkaloid per mg of tissue; error bars representing standard deviation. Sample dilutions were as follows: 10 fold for tissue culture samples, 1000 fold for flower, 5000 fold for leaf and stem, and 10000 fold for root, bulb, and rhizome. TCWK1 and TCWK2 stand for tissue culture one- and two- weeks after transfer to new media, respectively.

[0014] Figure 3. GC-MS Overlay of *S. frugiperda* Sf9 extracts expressing *V. californicum* genes. Extracts of *S. frugiperda* Sf9 cells infected with several combinations of Baculovirus containing genes from *V. californicum* were extracted and analyzed by gas chromatography mass spectrometry. Each colored chromatograph corresponds to the following: **Red**-control cytochrome P450 + CPR, **Orange**-cholesterol 22-hydroxylase + CPR, **Green**-cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase + CPR, **Blue**-cholesterol 22-hydroxylase + 22-hydroxy-26-aminocholesterol 22-oxidase + CPR, **Purple**-cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase + 22-hydroxy-26-aminocholesterol 22-oxidase + CPR. Metabolites are numbered according to the legend and shaded for clarity. CPR refers to the cytochrome P450 reductase from *Eschscholzia californica* and control P450 refers to CYP719A14 cheilanthifoline synthase from A*rgemone mexicana.*

[0015] Figure 4. LC-MS/MS of *S. frugiperda* Sf9 extracts expressing *V. californicum* genes. *S. frugiperda* Sf9 cells infected with several combinations of Baculovirus containing select genes from *V. californicum* were extracted and analyzed by liquid chromatography mass spectrometry with method stated above. (A) Extract of *S. frugiperda* Sf9 infection with cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxycholesterol-26-al transaminase, and CPR, (B) extract of *S. frugiperda* Sf9 infection with cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxy-26-aminocholesterol 22-oxidase, and CPR, (C) extract of *S. frugiperda* Sf9 infection with cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxycholesterol-26-al transaminase, 22-hydroxy-26-aminocholesterol 22-oxidase, and CPR, (D) extract of *S. frugiperda* Sf9 infection with cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxy-26-aminocholesterol 22-oxidase, *S. lycopersicum* GABA transaminase isozyme 2, and CPR. MRM signals for each metabolite

were combined and shaded for clarity. CPR refers to *E. californica* cytochrome P450 reductase.

[0016] Figure 5. Proposed *V. californicum* cyclopamine biosynthetic pathway leading from cholesterol. Cholesterol is first hydroxylated at position C-22 in the *R*-orientation by cholesterol 22-hydroxylase, followed by hydroxylation/oxidation at position C-26 by 22-hydroxycholesterol 26-hydroxylase/oxidase. Next, a transamination reaction by 22-hydroxycholesterol-26-al transaminase transfers an amino group from γ-aminobutyric acid to the C-26-aldehyde, forming 22-hydroxy-26-aminocholesterol. The C-22-hydroxy group is then oxidized to a ketone by 22-hydroxy-26-aminocholesterol 22-oxidase to form 22-keto-26-aminocholesterol, a reactive intermediate that quickly cyclizes to verazine.

[0017] Figure 6. Relative accumulation of select *V. californicum* metabolites and transcript abundance of biosynthetic genes per tissue type. Relative quantities of metabolites are shown by percent of total peak area, error bars show standard deviation for three replicates as determined by LC-MS/MS; quantities of transcript abundance are represented by percent of total reads. (A) A comparison of relative amounts of 22-keto-26-hydroxycholesterol and verazine to 22-hydroxy-26-aminocholesterol 22-oxidase, and (B) a comparison of 22-hydroxy-26-aminocholesterol to 22-hydroxycholesterol-26-al transaminase as determined by liquid chromatography mass spectrometry (metabolites) and read alignments to assembled transcriptome (gene transcripts). TCWK1 and TCWK2 stand for tissue culture one- and two-weeks after transfer for new media, respectively.

[0018] Figure 7. Cyclopamine accumulation *vs* gene expression of steroidal alkaloid biosynthetic genes. The presented tissues types from *V. californicum* were extracted and analyzed by liquid chromatography mass spectrometry for cyclopamine amount. Transcript abundance was analyzed by alignment of individual reads to the assembled transcriptome for gene expression. Both gene expression and cyclopamine accumulation are shown as a percent of the total for comparison. The abbreviation TC1WK and TC2WK stands for tissue culture one and two weeks after transfer to fresh media (respectively).

[0019] Figure 8. Phylogenetic tree of select plant cytochrome P450 enzymes. Nucleotide sequences obtained from Genbank, Uniprot, and the Sol Genomics Network of select cytochrome P450 enzymes were aligned by codon with the Muscle algorithm. Cytochrome P450 designations, species, and their corresponding function can be found in Table 11. Only experimentally determined functions are designated in the figure. Phylogenetic reconstruction

was performed using the Maximum likelihood statistical method with bootstrapping in MEGA version 6.06 with default parameters.

[0020] Figure 9. Phylogenetic tree of select γ-aminobutyrate transaminases (GABATS). Nucleotide sequences obtained from Genbank and Dendrome of select GABA-transaminases were aligned with the Muscle algorithm. GABA-transaminase designations, species and their accession numbers can be found in Table 12. Phylogenetic reconstruction was performed using the Maximum likelihood statistical method with bootstrapping in MEGA version 6.06 with default parameters.

[0021] Figure 10. Neighbor-joining phylogeny of CYP90B1 members. Candidate genes from Haystack are highlighted in red.

[0022] Figure 11. Enzyme Assays for biosynthetic pathway order clarification in *V. californicum* using LC-MS/MS. Enzyme assays were performed as described in Figure 1B using crude *S. frugiperda* Sf9 cells infected with baculovirus containing each *V. californicum* gene. All cytochromes P450 were co-expressed with *E. californica* cytochrome P450 reductase (CPR); 22-hydroxycholesterol-26-al transaminase was expressed alone. Cholesterol substrate for each initial reaction was provided by *S. frugiperda* Sf9 cells. Extracts were taken at each step and analyzed by liquid chromatography mass spectrometry. Chromatographs for each sample are a combination of product ions for clarity. (A) Chromatograms of assays run initially with cholesterol 22-hydroxylase combined with 22-hydroxycholesterol 26-hydroxylase/oxidase. Each colored chromatograph corresponds to the following: **Red**-cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase, **Orange**-cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase, extracted, then added as substrate to assay with 22-hydroxycholesterol-26-al transaminase, **Green**-cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase, extracted, then added as substrate to assay with 22-hydroxycholesterol-26-al transaminase, extracted, and added as substrate to 22-hydroxy-26-aminocholesterol 22-oxidase, **Blue**-cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase extracted and added as substrate to 22-hydroxy-26-aminocholesterol 22-oxidase, **Purple**-cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase, extracted, and added as substrate to 22-hydroxy-26-aminocholesterol 22-oxidase, extracted, and added as substrate to 22-hydroxycholesterol-26-al transaminase, **Grey**-CPR only control. (B) Chromatograms of assays run initially with cholesterol 22-hydroxylase + 22-hydroxy-26-aminocholesterol 22-

oxidase. Each colored chromatogram corresponds to the following: **Red**-cholesterol 22-hydroxylase + 22-hydroxy-26-aminocholesterol 22-oxidase, **Orange**-cholesterol 22-hydroxylase + 22-hydroxy-26-aminocholesterol 22-oxidase, extracted, and added as substrate to 22-hydroxycholesterol-26-al transaminase, **Green**-cholesterol 22-hydroxylase + 22-hydroxy-26-aminocholesterol 22-oxidase, extracted, and added as substrate to 22-hydroxycholesterol-26-al transaminase, extracted, then added as substrate to 22-hydroxycholesterol 26-hydroxylase/oxidase, **Blue**-cholesterol 22-hydroxylase + 22-hydroxy-26-aminocholesterol 22-oxidase, extracted, and added as substrate to 22-hydroxycholesterol 26-hydroxylase/oxidase, **Purple**-cholesterol 22-hydroxylase + 22-hydroxy-26-aminocholesterol 22-oxidase, extracted, and added as substrate to 22-hydroxycholesterol 26-hydroxylase/oxidase, extracted, then added as substrate to 22-hydroxycholesterol-26-al transaminase, **Grey**-CPR only control.

[0023] Figure 12. Orbitrap structural analysis. Purified (A) 22-hydroxy-26-aminocholesterol and (B) verazine were analyzed by high-resolution mass spectrometry for structural identification. Key fragments for each are shown.

[0024] Figure 13. SDS-PAGE of heterologously expressed *V. californicum* cytochromes P450 in *S. frugiperda* Sf9 cells. *S. frugiperda* Sf9 cell extracts expressing *V. californicum* cytochromes P450 co-expressed with *E. californica* cytochrome P450 reductase (CPR) was analyzed by SDS-PAGE and visualized by Coomassie Blue staining. (A) Lane 1, protein standards; lane 2-4, independent preparations of cholesterol 22-hydroxylase; lane 5, *S. frugiperda* Sf9 cell expression CPR only; lane 6, 0.3 μg bovine serum albumin (BSA); lane 7, 0.6 μg BSA; lane 8, 1.2 μg BSA; lane 9, 1.8 μg BSA; lane 10, replicate of lane 2. (B) Lane 1, loading buffer only; lane 2, 1.8 μg BSA; lane 3, 1.2 μg BSA; lane 4, 0.6 μg BSA; lane 5, loading buffer only; lane 6-8, independent preparations of 22-hydroxycholesterol 26-hydroxylase/oxidase; lane 9, CPR only; lane 10, protein standards. (C) Lane 1, loading buffer only; lane 2, 0.6 μg BSA; lane 3, 1.2 μg BSA; lane 4, 1.8 μg BSA; lane 5, CPR only; lane 6-9, independent preparations of 22-hydroxy-26-aminocholesterol 22-oxidase; lane 10, protein standards.

[0025] Figure 14. Expression profile of *V. californicum* genes involved in the biosynthesis of steroidal alkaloids from Illumina RNAseq and semi-quantitative RT-PCR. Relative abundance is based on percent of the total for each gene, normalized to the total number of reads or total density. (A) Cleaned Illumina reads were mapped to assembled

contigs to obtain alignment data. (B) Band density of semi-quantitative RT-PCR was used for quantitation.

[0026] Figure 15. GC-MS analysis of select *V. californicum* cytochrome P450 enzymes with cholesterol. Enzyme assays were extracted and derivatized before GC-MS analysis. (A) Overlay of enzyme assays performed with either cholesterol 22-hydroxylase or 22-hydroxy-26-aminocholesterol 22-oxidase using cholesterol as substrate. Both cytochrome P450 enzymes were co-expressed with *E. californica* cytochrome P450 reductase (CPR). 22(*R*)-Hydroxycholesterol pure standard was included for reference. (B) Enzyme assay of 22-hydroxycholesterol 26-hydroxylase/oxidase co-expressed with CPR with cholesterol as substrate overlain with pure 26-hydroxycholesterol for reference.

[0027] Figure 16. Enzyme assays for biosynthetic pathway order clarification in *V. californicum* using GC-MS. Assays were completed according to Figure 1A and analyzed by gas chromatography mass spectrometry. (A) Assay with control cytochrome P450 co-expressed with *E. californica* cytochrome P450 reductase (CPR) and authentic 22(*R*)-hydroxycholesterol was extracted, dried, and used as substrate in an assay with the same control cytochrome P450 + CPR. (B) Assay with 22-hydroxy-26-aminocholesterol 22-oxidase + CPR and 22(*R*)-hydroxycholesterol was extracted and used as substrate in assay with control cytochrome P450 + CPR. (C) Assay with 22-hydroxy-26-aminocholesterol 22-oxidase + CPR and 22(*R*)-hydroxycholesterol was extracted and used as substrate in assay with 22-hydroxycholesterol 26-hydroxylase/oxidase + CPR. (D) Assay with 22-hydroxycholesterol 26-hydroxylase/oxidase + CPR was extracted and used as substrate in assay with control cytochrome P450 + CPR. (E) Assay with 22-hydroxycholesterol 26-hydroxylase/oxidase + CPR and 22(*R*)-hydroxycholesterol was extracted and used as substrate in assay with 22-hydroxy-26-aminocholesterol 22-oxidase + CPR. (F) Schematic of possible transformation order; red X indicating which reaction was not observed. Ion 187 was extracted for the presented chromatograms.

[0028] Figure 17. Dimedone aldehyde trapping experiment. *S. frugiperda* Sf9 cell extracts expressing *V. californicum* cholesterol 22-hydroxylase co-expressed with *E. californica* cytochrome P450 reductase (CPR) and *V. californicum* 22-hydroxycholesterol 26-hydroxylase/oxidase co-expressed with CPR were mixed for enzyme assay using cholesterol provided by the crude cell extract as substrate. Assays performed without dimedone (blue) and with dimedone (red) were analyzed by liquid chromatography mass spectrometry.

Chromatograms were obtained by overlay of Enhanced Product Ion scans (EPI) for molecular mass 417.

[0029] Figure 18. Borohydride reduction of verazine. *S. frugiperda* Sf9 cells expressing the *V. californicum* genes cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxycholesterol-26-al transaminase, 22-hydroxy-26-aminocholesterol 22-oxidase, and *E. californica* cytochrome P450 reductase were extracted and analyzed by liquid chromatography mass spectrometry either directly (blue) or after treatment with $NaBH_4$ (red). Enhance MS scans detecting ions 380-425 m/z are presented.

[0030] Figure 19. LC-MS/MS analysis of *V. californicum* contig 674 (GABA transaminase 2). *S. frugiperda* Sf9 cells were co-transformed with *E. californica* cytochrome P450 reductase, *V. californicum* cytochrome P450 enzymes: cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxy-26-aminocholesterol 22-oxidase, and (A) Contig 674 or (B) 22-hydroxycholesterol-26-al transaminase. Extracts were analyzed by LC-MS/MS; ions for each peak were combined and shaded for clarity.

[0031] Figure 20. 22-Keto-26-hydroxycholesterol in refactored *S. frugiperda* Sf9 cells and *Camelina sativa.* Extracts were analyzed by liquid chromatography mass spectrometry using MRM mode for ion 417/271. **Blue**-wild type camelina seed extract, **Red**-transgenic camelina seed extract expressing cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxy-26-aminocholesterol 22-oxidase, and 22-hydroxycholesterol-26-al transaminase, **Green**-*S. frugiperda* Sf9 cells expressing cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxy-26-aminocholesterol 22-oxidase, 22-hydroxycholesterol-26-al transaminase, CPR.

[0032] Figure 21. Production of verazine by heterologous expression of *Veratrum californica* genes in *S. frugiperda* Sf9 cells. Select genes were introduced into *S. frugiperda* Sf9 cells using a baculovirus expression system. Metabolites were extracted and analyzed by LC-MS/MS in the full scan Enhanced MS mode detecting masses 380 – 425. Each colored chromatogram represent the combination of genes as follows: Green-Cholesterol 22-hydroxylase, 22-Hydroxycholesterol 26-hydroxylase/oxidase, 22-Hydroxy-26-aminocholesterol 22-oxidase, and CPR; Orange-Cholesterol 22-hydroxylase, 22-Hydroxycholesterol 26-hydroxylase/oxidase, 22-Hydroxy-26-aminocholesterol 22-oxidase, 22-Hydroxycholesterol-26-al transaminase, CPR; Pink-No enzyme control. CPR refers to the cytochrome P450 reductase from *Eschscholzia californica*. Peak at 398.4 is verazine, peak at

417.2 is 22-keto-26-hydroxycholesterol, and peak 418.4 is for 22-hydroxy-26-aminocholesterol.

[0033] Figure 22. Mass spectra of select derivatized standards and enzymatically formed products. Enzyme assays using recombinant Veratrum californicum genes and authentic standards were first extracted with hexane and derivatized with Sylon HTP before GC-MS analysis. (a) Spectrum of 22(R)-hydroxycholesterol produced by enzyme assay using S. frugiperda Sf9 cells expressing CYP90B27 (cholesterol 22-hydroxylase) and E. californica cytochrome P450 reductase (CPR) with pure cholesterol as substrate. (b) Spectrum of 22-keto-cholesterol produced by enzyme assay using Sf9 cells expressing CYP90G1 (22-hydroxy-26-aminocholesterol 22-oxidase) with pure 22(R)-hydroxycholesterol as substrate. (c) Spectrum of pure 7β-hydroxycholesterol. (d) Spectrum of pure 26-hydroxycholesterol. (e) Spectrum of 7β,22-dihydroxycholesterol produced by enzyme assay using Sf9 cells expressing CYP90B27 and CPR with pure 7β-hydroxycholesterol as substrate. (f) Spectrum of 22-keto-26-hydroxycholesterol produce by infection of Sf9 cells with CYP90B27, CYP90G1, CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase), and CPR. Mass spectra after background subtraction is shown.

[0034] Figure 23. GC-MS analysis of Veratrum californicum CYP90B27 with 26-hydroxycholesterol and 7β-hydroxycholesterol. Enzyme assays using S. frugiperda Sf9 cells expressing CYP90B27 (cholesterol 22-hydroxylase) and E. californica cytochrome P450 reductase (CPR) were extracted and derivatized with Sylon HTP before GC-MS analysis. (a) Enzyme assays using 26-hydroxycholesterol [27(25R)-hydroxycholesterol] as substrate. 26-Hydroxycholesterol [27(25R)-hydroxycholesterol] pure standard was included for reference. Control Sf9 cells expressing CYP719A14, cheilanthifoline synthase from Argemone mexicana (an unrelated cytochrome P450), and CPR, were run in parallel with 26-hydroxycholesterol [27(25R)-hydroxycholesterol] as a control. (b) Enzyme assays with 7β-hydroxycholesterol as substrate. 7β-Hydroxycholesterol pure standard was included for reference and Sf9 cells expressing CYP719A14 and CPR were run in parallel with 7β -hydroxycholesterol as a control.

[0035] Figure 24. GC-MS analysis of Veratrum californicum cytochrome P450 enzymes CYP90B27 and CYP90G1. S. frugiperda Sf9 cells expressing enzyme CYP90B27 (cholesterol 22-hydroxylase), CYP90G1 (22-hydroxy-26-aminocholesterol 22-oxidase), and E. californica cytochrome P450 reductase (CPR) were extracted and derivatized with Sylon

HTP before GC-MS analysis. 22(R)-Hydroxycholesterol and 22(S)-hydroxycholesterol pure standards were included for reference. Control cells expressing CYP719A14, cheilanthifoline synthase from A. mexicana (an unrelated cytochrome P450), and CPR were also assayed with 22(R)-hydroxycholesterol as a control.

[0036] Figure 25. LC-MS/MS of enzyme assays with his-tag purified GABAT1. Recombinant GABAT1 from Veratrum californicum was his-tag purified from E.coli PLUS E cells and used in enzyme assays with the substrate 22-hydroxycholesterol-26-al and GABA, L-arginine, or L-glutamine to determine the amino group donor. Sf9 cells infected with CPR were used as a negative control, and Sf9 cells infected with GABAT1 were used as a positive control. MRM signal 418/400 is presented for each assay. CPR refers to E. californica cytochrome P450 reductase. Each assay was performed in duplicate, with one representative chromatogram shown.

DESCRIPTION

[0037] The following detailed description is provided to aid those skilled in the art. Even so, the following detailed description should not be construed to unduly limit, as modifications and variations in the embodiments discussed herein may be made by those of ordinary skill in the art without departing from the spirit or scope of the present disclosure.

[0038] Any feature, or combination of features, described herein is (are) included within the scope of the present disclosure, provided that the features included in any such combination are not mutually inconsistent as will be apparent from the context, this specification, and the knowledge of one of ordinary skill in the art. Additional advantages and aspects of the present disclosure are apparent in the following detailed description and claims.

[0039] The contents of each of the publications, patent applications, patents, and other references mentioned herein are incorporated by reference in their entirety. In case of conflict, the present disclosure, including explanations of terms, will control.

I. Terms

[0040] The following definitions are provided to aid the reader in understanding the various aspects of the present disclosure. Unless defined otherwise, all technical and scientific terms used herein have the same meaning as commonly understood by those of ordinary skill in the art to which the disclosure pertains. Units, prefixes and symbols may be denoted in their SI

accepted form. Provision, or lack of the provision, of a definition for a particular term or phrase is not meant to signify any particular importance, or lack thereof. Rather, and unless otherwise noted, terms used and the manufacture or laboratory procedures described herein are well known and commonly employed in the art. Conventional methods are used for these procedures, such as those provided in the art and various general references.

[0041] As used herein and in the appended claims, the singular forms “a”, “an”, and “the” include plural reference unless the context clearly dictates otherwise. Thus, for example, reference to “a plant” includes a plurality of such plants, reference to “a cell” includes one or more cells and equivalents thereof known to those skilled in the art, and so forth. Similarly, the word "or" is intended to include "and" unless the context clearly indicates otherwise. Hence "comprising A or B" means including A, or B, or A and B. Furthermore, the use of the term “including”, as well as other related forms, such as “includes” and “included”, is not limiting.

[0042] The term “comprising” as used in a claim herein is open-ended, and means that the claim must have all the features specifically recited therein, but that there is no bar on additional features that are not recited being present as well. The term “comprising” leaves the claim open for the inclusion of unspecified ingredients even in major amounts. The term “consisting essentially of” in a claim means that the invention necessarily includes the listed ingredients, and is open to unlisted ingredients that do not materially affect the basic and novel properties of the invention. A “consisting essentially of” claim occupies a middle ground between closed claims that are written in a closed “consisting of” format and fully open claims that are drafted in a “comprising’ format”. These terms can be used interchangeably herein if, and when, this may become necessary. Furthermore, the use of the term “including”, as well as other related forms, such as “includes” and “included”, is not limiting.

[0043] Unless otherwise stated, nucleic acid sequences in the text of this specification are given, when read from left to right, in the 5' to 3' direction. Nucleic acid sequences may be provided as DNA or as RNA, as specified; disclosure of one necessarily defines the other, as is known to one of ordinary skill in the art and is understood as included in embodiments where it would be appropriate. Nucleotides may be referred to by their commonly accepted single-letter codes. Unless otherwise indicated, amino acid sequences are written left to right in amino to carboxyl orientation, respectively. Amino acids may be referred to herein by

either their commonly known three letter symbols or by the one-letter symbols recommended by the IUPAC-IUM Biochemical Nomenclature Commission. It is further to be understood that all base sizes or amino acid sizes, and all molecular weight or molecular mass values, given for nucleic acids or polypeptides are approximate, and are provided for description purposes and are not to be unduly limiting. Unless otherwise provided for, software, electrical, and electronics terms as used herein are as defined in The New IEEE Standard Dictionary of Electrical and Electronics Terms (5th edition, 1993). The terms defined below are more fully defined by reference to the specification as a whole.

[0044] If ranges are disclosed, the endpoints of all ranges directed to the same component or property are inclusive and independently combinable (e.g., ranges of "up to about 25 wt.%, or, more specifically, about 5 wt.% to about 20 wt.%," is inclusive of the endpoints and all intermediate values of the ranges of "about 5 wt.% to about 25 wt.%," etc.). Numeric ranges recited with the specification are inclusive of the numbers defining the range and include each integer within the defined range.

[0045] The term **"about"** as used herein is a flexible word with a meaning similar to "approximately" or "nearly". The term "about" indicates that exactitude is not claimed, but rather a contemplated variation. Thus, as used herein, the term "about" means within 1 or 2 standard deviations from the specifically recited value, or ± a range of up to 20%, up to 15%, up to 10%, up to 5%, or up to 4%, 3%, 2%, or 1% compared to the specifically recited value.

[0046] As used herein, "**altering level of production**" or "**altering level of expression**" means changing, either by increasing or decreasing, the level of production or expression of a nucleic acid sequence or an amino acid sequence (for example a polypeptide, an siRNA, a miRNA, an mRNA, a gene), as compared to a control level of production or expression.

[0047] The phrase "**conservative amino acid substitution**" or "**conservative mutation**" refers to the replacement of one amino acid by another amino acid with a common property. A functional way to define common properties between individual amino acids is to analyze the normalized frequencies of amino acid changes between corresponding proteins of homologous organisms (Schulz, G. E. and R. H. Schirmer (1979) Principles of Protein Structure, Springer-Verlag). According to such analyses, groups of amino acids can be defined where amino acids within a group exchange preferentially with each other, and therefore resemble each other most in their impact on the overall protein structure.

[0048] Examples of amino acid groups defined in this manner include: a "charged / polar group," consisting of Glu, Asp, Asn, Gln, Lys, Arg and His; an "aromatic, or cyclic group," consisting of Pro, Phe, Tyr and Trp; and an "aliphatic group" consisting of Gly, Ala, Val, Leu, Ile, Met, Ser, Thr and Cys. Within each group, subgroups can also be identified, for example, the group of charged / polar amino acids can be sub-divided into the sub-groups consisting of the "positively-charged sub-group," consisting of Lys, Arg and His; the negatively-charged sub-group," consisting of Glu and Asp, and the "polar sub-group" consisting of Asn and Gln. The aromatic or cyclic group can be sub-divided into the sub-groups consisting of the "nitrogen ring sub-group," consisting of Pro, His and Trp; and the "phenyl sub-group" consisting of Phe and Tyr. The aliphatic group can be sub-divided into the sub-groups consisting of the "large aliphatic non-polar sub-group," consisting of Val, Leu and Ile; the "aliphatic slightly-polar sub-group," consisting of Met, Ser, Thr and Cys; and the "small-residue sub-group," consisting of Gly and Ala. Examples of conservative mutations include substitutions of amino acids within the sub-groups above, for example, Lys for Arg and vice versa such that a positive charge can be maintained; Glu for Asp and *vice versa* such that a negative charge can be maintained; Ser for Thr such that a free -OH can be maintained; and Gln for Asn such that a free -NH_2 can be maintained.

[0049] As used herein "**control**" or "**control level**" means the level of a molecule, such as a polypeptide or nucleic acid, normally found in nature under a certain condition and/or in a specific genetic background. In certain embodiments, a control level of a molecule can be measured in a cell or specimen that has not been subjected, either directly or indirectly, to a treatment. A control level is also referred to as a wildtype or a basal level. These terms are understood by those of ordinary skill in the art. A control plant, i.e. a plant that does not contain a recombinant DNA that confers (for instance) an enhanced trait in a transgenic plant, is used as a baseline for comparison to identify an enhanced trait in the transgenic plant. A suitable control plant may be a non-transgenic plant of the parental line used to generate a transgenic plant. A control plant may in some cases be a transgenic plant line that comprises an empty vector or marker gene, but does not contain the recombinant DNA, or does not contain all of the recombinant DNAs in the test plant.

[0050] The terms "**enhance**", "**enhanced**", "**increase**", or "**increased**" refer to a statistically significant increase. For the avoidance of doubt, these terms generally refer to about a 5% increase in a given parameter or value, about a 10% increase, about a 15% increase, about a 20% increase, about a 25% increase, about a 30% increase, about a 35% increase, about a

40% increase, about a 45% increase, about a 50% increase, about a 55% increase, about a 60% increase, about a 65% increase, about 70% increase, about a 75% increase, about an 80% increase, about an 85% increase, about a 90% increase, about a 95% increase, about a 100% increase, or more over the control value. These terms also encompass ranges consisting of any lower indicated value to any higher indicated value, for example "from about 5% to about 50%", etc.

[0051] As used herein, "**expression**" or "**expressing**" refers to production of a functional product, such as, the generation of an RNA transcript from an introduced construct, an endogenous DNA sequence, or a stably incorporated heterologous DNA sequence. A nucleotide encoding sequence may comprise intervening sequence (e.g. introns) or may lack such intervening non-translated sequences (e.g. as in cDNA). Expressed genes include those that are transcribed into mRNA and then translated into protein and those that are transcribed into RNA but not translated (for example, siRNA, transfer RNA and ribosomal RNA). The term may also refer to a polypeptide produced from an mRNA generated from any of the above DNA precursors. Thus, expression of a nucleic acid fragment, such as a gene or a promoter region of a gene, may refer to transcription of the nucleic acid fragment (e.g., transcription resulting in mRNA or other functional RNA) and/ or translation of RNA into a precursor or mature protein (polypeptide), or both.

[0052] An **"expression cassette"** refers to a nucleic acid construct, which when introduced into a host cell, results in transcription and/or translation of a RNA or polypeptide, respectively.

[0053] The term "**genome**" as it applies to a plant cells encompasses not only chromosomal DNA found within the nucleus, but organelle DNA found within subcellular components (e.g., mitochondrial, plastid) of the cell. As used herein, the term "genome" refers to the nuclear genome unless indicated otherwise. However, expression in a plastid genome, e.g., a chloroplast genome, or targeting to a plastid genome such as a chloroplast via the use of a plastid targeting sequence, is also encompassed by the present disclosure.

[0054] A polynucleotide sequence is **"heterologous to"** a second polynucleotide sequence if it originates from a foreign species, or, if from the same species, is modified by human action from its original form. For example, a promoter operably linked to a heterologous coding sequence refers to a coding sequence from a species different from that from which the promoter was derived, or, if from the same species, a coding sequence which is different from

naturally occurring allelic variants. Heterologous nucleic acid fragments, such as coding sequences that have been inserted into a host organism, are not normally found in the genetic complement of the host organism. As used herein, the term "heterologous" also refers to a nucleic acid fragment derived from the same organism, but which is located in a different, e.g., non-native, location within the genome of this organism. Thus, the organism can have more than the usual number of copy(ies) of such nucleic acid fragment located in its(their) normal position within the genome and in addition, in the case of plant cells, within different genomes within a cell, for example in the nuclear genome and within a plastid or mitochondrial genome as well. A nucleic acid fragment that is heterologous with respect to an organism into which it has been inserted or transferred is sometimes referred to as a "transgene."

[0055] The term "**homology**" describes a mathematically based comparison of sequence similarities which is used to identify genes or proteins with similar functions or motifs. The nucleic acid and protein sequences of the present invention can be used as a "query sequence" to perform a search against public databases to, for example, identify other family members, related sequences or homologs. The term **"homologous**" refers to the relationship between two nucleic acid sequence and/or proteins that possess a "common evolutionary origin", including nucleic acids and/or proteins from superfamilies (e.g., the immunoglobulin superfamily) in the same species of animal, as well as homologous nucleic acids and/or proteins from different species of animal (for example, myosin light chain polypeptide, etc.; see Reeck et al., (1987) *Cell,* 50:667). Such proteins (and their encoding nucleic acids) may have sequence homology, as reflected by sequence similarity, whether in terms of percent identity or by the presence of specific residues or motifs and conserved positions. The methods disclosed herein contemplate the use of the presently disclosed nucleic and protein sequences, as well as sequences having sequence identity and/or similarity.

[0056] By **"host cell"** it is meant a cell which contains a vector and supports the replication and/or expression of the vector. Host cells may be prokaryotic cells such as *E. coli*, or eukaryotic cells such as yeast, insect, amphibian, or mammalian cells. Alternatively, the host cells are monocotyledonous or dicotyledonous plant cells.

[0057] The term "**introduced**" means providing a nucleic acid (e.g., expression construct) or protein into a cell. Introduced includes reference to the incorporation of a nucleic acid into a eukaryotic or prokaryotic cell where the nucleic acid may be incorporated into the genome of

the cell, and includes reference to the transient provision of a nucleic acid or protein to the cell. "Introduced" includes reference to stable or transient transformation methods, as well as sexually crossing. Thus, "introduced" in the context of inserting a nucleic acid fragment (e.g., a recombinant DNA construct/expression construct) into a cell, can mean "transfection" or "transformation" or "transduction", and includes reference to the incorporation of a nucleic acid fragment into a eukaryotic or prokaryotic cell where the nucleic acid fragment may be incorporated into the genome of the cell (e.g., chromosome, plasmid, plastid or mitochondrial DNA), converted into an autonomous replicon, or transiently expressed (e.g., transfected mRNA).

[0058] As used herein the term "**isolated**" refers to a material such as a nucleic acid molecule, polypeptide, or small molecule, such as cyclopamine, that has been separated from the environment from which it was obtained. It can also mean altered from the natural state. For example, a polynucleotide or a polypeptide naturally present in a living animal is not "isolated" but the same polynucleotide or polypeptide separated from the coexisting materials of its natural state is "isolated", as the term is employed herein. Thus, a polypeptide or polynucleotide produced and/or contained within a recombinant host cell is considered isolated. Also intended as "isolated polypeptides" or "isolated nucleic acid molecules", etc., are polypeptides or nucleic acid molecules that have been purified, partially or substantially, from a recombinant host cell or from a native source.

[0059] As used here "**modulate**" or "**modulating**" or "**modulation**" and the like are used interchangeably to denote either up-regulation or down-regulation of the expression or biosynthesis of a material such as a nucleic acid, protein or small molecule relative to its normal expression or biosynthetic level in a wild type or control organism. Modulation includes expression or biosynthesis that is increased or decreased by about 5%, 10%, 15%, 20%, 25%, 30%, 35%, 40%, 45%, 50%, 55%, 60%, 65%, 70%, 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 99.1%, 99.5%, 99.9%, 100%, 110%, 115%, 120%, 125%, 130%, 135%, 140%, 145%, 150% , 155%, 160%, 165% or 170% or more relative to the wild type or control expression or biosynthesis level. As described herein, various material accumulation, such as that of cyclopamine, can be increased, or in the case of some embodiments, sometimes decreased relative to a control. One of ordinary skill will be able to identify or produce a relevant control.

[0060] As used herein, "**nucleic acid**" means a polynucleotide (or oligonucleotide), including single or double-stranded polymers of deoxyribonucleotide or ribonucleotide bases, and unless otherwise indicated, encompasses naturally occurring and synthetic nucleotide analogues having the essential nature of natural nucleotides in that they hybridize to complementary single-stranded nucleic acids in a manner similar to naturally occurring nucleotides. Nucleic acids may also include fragments and modified nucleotide sequences. Nucleic acids disclosed herein can either be naturally occurring, for example genomic nucleic acids; or isolated, purified, non-genomic nucleic acids, including synthetically produced nucleic acid sequences such as those made by chemical oligonucleotide synthesis, enzymatic synthesis, or by recombinant methods, including for example, cDNA, codon-optimized sequences for efficient expression in different transgenic plants reflecting the pattern of codon usage in such plants, nucleotide sequences that differ from the nucleotide sequences disclosed herein due to the degeneracy of the genetic code but that still encode the protein(s) of interest disclosed herein, nucleotide sequences encoding the presently disclosed protein(s) comprising conservative (or non-conservative) amino acid substitutions that do not adversely affect their normal activity, PCR-amplified nucleotide sequences, and other non-genomic forms of nucleotide sequences familiar to those of ordinary skill in the art.

[0061] As used herein, "**nucleic acid construct**" or "**construct**" refers to an isolated polynucleotide which can be introduced into a host cell. This construct may comprise any combination of deoxyribonucleotides, ribonucleotides, and/or modified nucleotides. This construct may comprise an expression cassette that can be introduced into and expressed in a host cell.

[0062] As used herein "**operably linked**" refers to a functional arrangement of elements. A first nucleic acid sequence is operably linked with a second nucleic acid sequence when the first nucleic acid sequence is placed in a functional relationship with the second nucleic acid sequence. For instance, a promoter is operably linked to a coding sequence if the promoter effects the transcription or expression of the coding sequence. The control elements need not be contiguous with the coding sequence, so long as they function to direct the expression thereof. Thus, for example, intervening untranslated yet transcribed sequences can be present between a promoter and the coding sequence and the promoter can still be considered "operably linked" to the coding sequence.

[0063] As used herein, the terms "**plant**" or "**plants**" that can be used in the present methods broadly include the classes of higher and lower plants amenable to transformation techniques, including angiosperms (monocotyledonous and dicotyledonous plants), gymnosperms, ferns, and unicellular and multicellular algae. The term "plant" also includes plants which have been modified by breeding, mutagenesis or genetic engineering (transgenic and non-transgenic plants). It includes plants of a variety of ploidy levels, including aneuploid, polyploid, diploid, haploid and hemizygous. The plant may be in any form including suspension cultures, embryos, meristematic regions, callus tissue, gametophytes, sporophytes, pollen, microspores, whole plants, shoot vegetative organs/structures (e.g. leaves, stems and tubers), roots, flowers and floral organs/structures, seed (including embryo, endosperm, and seed coat) and fruit, plant tissue (e.g. vascular tissue, ground tissue, and the like) and cells, and progeny of same. The term "**food crop plant**" includes plants that are either directly edible, or which produce edible products, and that are customarily used to feed humans either directly, or indirectly through animals. Non-limiting examples of such plants include: Cereal crops: wheat, rice, maize (corn), barley, oats, sorghum, rye, and millet; Protein crops: peanuts, chickpeas, lentils, kidney beans, soybeans, lima beans; Roots and tubers: potatoes, sweet potatoes, and cassavas; Oil crops: corn, soybeans, canola (rapeseed), wheat, peanuts, palm, coconuts, safflower, sesame, cottonseed, sunflower, flax, olive, and safflower; Sugar crops: sugar cane and sugar beets; Fruit crops: bananas, oranges, apples, pears, breadfruit, pineapples, and cherries; Vegetable crops and tubers: tomatoes, lettuce, carrots, melons, asparagus, etc.; Nuts: cashews, peanuts, walnuts, pistachio nuts, almonds; Forage and turf grasses; Forage legumes: alfalfa, clover; Drug crops: coffee, cocoa, kola nut, poppy, tobacco; Spice and flavoring crops: vanilla, sage, thyme, anise, saffron, menthol, peppermint, spearmint, coriander. The terms **"biofuels crops"**, **"energy crops"**, **"oil crops"**, **"oilseed crops",** and the like, to which the present methods and compositions can also be applied include the oil crops and further include plants such as sugarcane, castor bean, *Camelina*, switchgrass, *Miscanthus*, and *Jatropha*, which are used, or are being investigated and/or developed, as sources of biofuels due to their significant oil production and accumulation.

[0064] The terms "**peptide**", "**polypeptide**", and "**protein**" are used to refer to polymers of amino acid residues. These terms are specifically intended to cover naturally occurring biomolecules, as well as those that are recombinantly or synthetically produced.

[0065] The term "**promoter**" or "**regulatory element**" refers to a region or nucleic acid sequence located upstream or downstream from the start of transcription and which is

involved in recognition and binding of RNA polymerase and/or other proteins to initiate transcription of RNA. Promoters need not be of plant or algal origin, for example, promoters derived from plant viruses, such as the CaMV35S promoter, or from other organisms, can be used in variations of the embodiments discussed herein. Promoters useful in the present methods include constitutive, tissue-specific, cell-type specific, seed-specific, inducible, repressible, and developmentally regulated promoters.

[0066] A skilled person appreciates that a promoter sequence can be modified to provide for a range of expression levels of an operably linked heterologous nucleic acid molecule. Less than the entire promoter region can be utilized and the ability to drive expression retained. However, it is recognized that expression levels of mRNA can be decreased with deletions of portions of the promoter sequence. Thus, the promoter can be modified to be a weak or strong promoter. A promoter is classified as strong or weak according to its affinity for RNA polymerase (and/or sigma factor); this is related to how closely the promoter sequence resembles the ideal consensus sequence for the polymerase. Generally, by "weak promoter" is intended a promoter that drives expression of a coding sequence at a low level. By "low level" is intended levels of about 1/10,000 transcripts to about 1/100,000 transcripts to about 1/500,000 transcripts. Conversely, a strong promoter drives expression of a coding sequence at a high level, or at about 1/10 transcripts to about 1/100 transcripts to about 1/1,000 transcripts. The promoter of choice is preferably excised from its source by restriction enzymes, but can alternatively be PCR-amplified using primers that carry appropriate terminal restriction sites. It should be understood that the foregoing groups of promoters are non-limiting, and that one skilled in the art could employ other promoters that are not explicitly cited herein.

[0067] The term "**purified**" refers to material such as a nucleic acid, a protein, or a small molecule, such as cyclopamine, which is substantially or essentially free from components which normally accompany or interact with the material as found in its naturally occurring environment, and/or which may optionally comprise material not found within the purified material's natural environment. The latter may occur when the material of interest is expressed or synthesized in a non-native environment. Nucleic acids and proteins that have been isolated include nucleic acids and proteins purified by standard purification methods. The term also embraces nucleic acids and proteins prepared by recombinant expression in a host cell as well as chemically synthesized nucleic acids. The present disclosure also encompasses methods and compositions comprising cyclopamine. In some embodiments, the

cyclopamine is purified for therapeutic use and is formulated as a pharmaceutical composition. Such pharmaceutical compositions can be prepared by methods well known in the art. See, e.g., *Remington: The Science and Practice of Pharmacy*, 21st Edition (2005), Lippincott Williams & Wilkins, Philadelphia, PA.

[0068] "**Recombinant**" refers to a nucleotide sequence, peptide, polypeptide, or protein, expression of which is engineered or manipulated using standard recombinant methodology. This term applies to both the methods and the resulting products. As used herein, a "**recombinant construct**", "**expression construct**", "**chimeric construct**", "**construct**" and "**recombinant expression cassette**" are used interchangeably herein.

[0069] As used herein, the phrase **"sequence identity"** or "**sequence similarity**" is the similarity between two (or more) nucleic acid sequences, or two (or more) amino acid sequences. Sequence identity is frequently measured as the percent of identical nucleotide or amino acid residues at corresponding positions in two or more sequences when the sequences are aligned to maximize sequence matching, i.e., taking into account gaps and insertions.

[0070] One of ordinary skill in the art will appreciate that sequence identity ranges are provided for guidance only. It is entirely possible that nucleic acid sequences that do not show a high degree of sequence identity can nevertheless encode amino acid sequences having similar functional activity. It is understood that changes in nucleic acid sequence can be made using the degeneracy of the genetic code to produce multiple nucleic acid molecules that all encode substantially the same protein. Means for making this adjustment are well-known to those of skill in the art. When percentage of sequence identity is used in reference to amino acid sequences it is recognized that residue positions which are not identical often differ by conservative amino acid substitutions, where amino acid residues are substituted for other amino acid residues with similar chemical properties (e.g., charge or hydrophobicity) and therefore do not change the functional properties of the molecule. Where sequences differ in conservative substitutions, the percent sequence identity may be adjusted upwards to correct for the conservative nature of the substitution. Sequences which differ by such conservative substitutions are said to have "sequence similarity" or "similarity". Means for making this adjustment are well-known to those of skill in the art. Typically this involves scoring a conservative substitution as a partial rather than a full mismatch, thereby increasing the percentage sequence identity.

[0071] "Percentage of sequence identity" is determined by comparing two optimally aligned sequences over a comparison window, wherein the portion of the polynucleotide sequence in the comparison window may comprise additions or deletions (i.e., gaps) as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. The percentage is calculated by determining the number of positions at which the identical nucleic acid base or amino acid residue occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison and multiplying the result by 100 to yield the percentage of sequence identity.

[0072] Sequence identity (or similarity) can be readily calculated by known methods, including but not limited to those described in: *Computational Molecular Biology*, Lesk, A. M., ed., Oxford University Press, New York, 1988; *Biocomputing: Informatics and Genome Projects*, Smith, D. W., ed., Academic Press, New York, 1993; *Computer Analysis of Sequence Data, Part I*, Griffin, A. M., and Griffin, H. G., eds., Humana Press, New Jersey, 1994; *Sequence Analysis in Molecular Biology*, von Heinje, G., Academic Press, 1987; and *Sequence Analysis Primer*, Gribskov, M. and Devereux, J., eds., M Stockton Press, New York, 1991; and Carillo, H., and Lipman, D., *SIAM J. Applied Math.*, 48: 1073 (1988). Methods to determine identity are designed to give the largest match between the sequences tested. Moreover, methods to determine identity are codified in publicly available computer programs. Optimal alignment of sequences for comparison can be conducted, for example, by the local homology algorithm of Smith & Waterman, by the homology alignment algorithms, by the search for similarity method or, by computerized implementations of these algorithms (GAP, BESTFIT, PASTA, and TFASTA in the GCG Wisconsin Package, available from Accelrys, Inc., San Diego, California, United States of America), or by visual inspection. See generally, (Altschul, S. F. et al., J. Mol. Biol. 215: 403-410 (1990) and Altschul et al. Nucl. Acids Res. 25: 3389-3402 (1997)).

[0073] One example of an algorithm that is suitable for determining percent sequence identity and sequence similarity is the BLAST algorithm, which is described in (Altschul, S., et al., NCBI NLM NIH Bethesda, Md. 20894; & Altschul, S., et al., J. Mol. Biol. 215: 403-410 (1990). Software for performing BLAST analyses is publicly available through the National Center for Biotechnology Information. This algorithm involves first identifying high scoring sequence pairs (HSPs) by identifying short words of length W in the query sequence, which either match or satisfy some positive-valued threshold score T when aligned with a

word of the same length in a database sequence. T is referred to as the neighborhood word score threshold. These initial neighborhood word hits act as seeds for initiating searches to find longer HSPs containing them. The word hits are then extended in both directions along each sequence for as far as the cumulative alignment score can be increased. Cumulative scores are calculated using, for nucleotide sequences, the parameters M (reward score for a pair of matching residues; always > 0) and N (penalty score for mismatching residues; always < 0). For amino acid sequences, a scoring matrix is used to calculate the cumulative score. Extension of the word hits in each direction are halted when: the cumulative alignment score falls off by the quantity X from its maximum achieved value, the cumulative score goes to zero or below due to the accumulation of one or more negative-scoring residue alignments, or the end of either sequence is reached. The BLAST algorithm parameters W, T, and X determine the sensitivity and speed of the alignment. The BLASTN program (for nucleotide sequences) uses as defaults a wordlength (W) of 11, an expectation (E) of 10, a cutoff of 100, M = 5, N = -4, and a comparison of both strands. For amino acid sequences, the BLASTP program uses as defaults a wordlength (W) of 3, an expectation (E) of 10, and the BLOSUM62 scoring matrix (see Henikoff & Henikoff(1989) Proc. Natl. Acad. Sci. USA 89:10915).

[0074] In addition to calculating percent sequence identity, the BLAST algorithm also performs a statistical analysis of the similarity between two sequences (see, e.g., Karlin & Altschul, Proc. Nat'l. Acad. Sci. USA 90: 5873-5877 (1993)). One measure of similarity provided by the BLAST algorithm is the smallest sum probability (P (N)), which provides an indication of the probability by which a match between two nucleotide or amino acid sequences would occur by chance. BLAST searches assume that proteins can be modeled as random sequences. However, many real proteins comprise regions of nonrandom sequences which may be homopolymeric tracts, short-period repeats, or regions enriched in one or more amino acids. Such low-complexity regions may be aligned between unrelated proteins even though other regions of the protein are entirely dissimilar. A number of low-complexity filter programs can be employed to reduce such low-complexity alignments. For example, the SEG (Wooten and Federhen, Comput. Chern., 17: 149-163 (1993)) and XNU (Claverie and States, Comput. Chern., 17: 191-201 (1993)) low-complexity filters can be employed alone or in combination.

[0075] The constructs and methods disclosed herein encompass nucleic acid and protein sequences having sequence identity/sequence similarity at least about 75%, 80%, 85%, 90%, 95%, 96%, 97%, 98%, 99%, 100% to those specifically disclosed.

[0076] A "**transgenic**" organism, such as a transgenic plant, is a host organism that has been stably or transiently genetically engineered to contain one or more heterologous nucleic acid fragments, including nucleotide coding sequences, expression cassettes, vectors, etc. Introduction of heterologous nucleic acids into a host cell to create a transgenic cell is not limited to any particular mode of delivery, and includes, for example, microinjection, adsorption, electroporation, particle gun bombardment, whiskers-mediated transformation, liposome-mediated delivery, *Agrobacterium*-mediated transfer, the use of viral and retroviral vectors, etc., as is well known to those skilled in the art.

[0077] Conventional techniques of molecular biology, recombinant DNA technology, microbiology, chemistry useful in practicing the methods of the present disclosure are described, for example, in Green and Sambrook (2012) *Molecular Cloning: A Laboratory Manual*, Fourth Edition, Cold Spring Harbor Laboratory Press; Ausubel et al. (2003 and periodic supplements) *Current Protocols in Molecular Biology*, John Wiley & Sons, New York, N.Y.; Amberg et al. (2005) *Methods in Yeast Genetics: A Cold Spring Harbor Laboratory Course Manual, 2005 Edition*, Cold Spring Harbor Laboratory Press; Roe et al. (1996) *DNA Isolation and Sequencing: Essential Techniques*, John Wiley & Sons; J. M. Polak and James O'D. McGee (1990) *In Situ Hybridization: Principles and Practice*; Oxford University Press; M. J. Gait (Editor) (1984) *Oligonucleotide Synthesis: A Practical Approach*, IRL Press; D. M. J. Lilley and J. E. Dahlberg (1992) *Methods in Enzymology: DNA Structure Part A: Synthesis and Physical Analysis of DNA*, Academic Press; and *Lab Ref: A Handbook of Recipes, Reagents, and Other Reference Tools for Use at the Bench*, Edited by Jane Roskams and Linda Rodgers (2002) Cold Spring Harbor Laboratory Press; Burgess and Deutscher (2009) *Guide to Protein Purification*, Second Edition (*Methods in Enzymology*, Vol. 463), Academic Press. Note also U.S. Patent Nos. 8,178,339; 8,119,365; 8,043,842; 8,039,243; 7,303,906; 6,989,265; US20120219994A1; and EP1483367B1. The entire contents of each of these texts and patent documents are herein incorporated by reference.

[0078] II. Overview of the Several Embodiments

[0079] In one embodiment, the invention relates to a transgenic plant or a transgenic organism that produces cyclopamine and/or verazine-derived metabolite. The transgenic plant or the transgenic organism, comprising within its genome, and expressing, a heterologous nucleotide sequence coding for one or more cytochrome P450 enzyme(s) and/or a γ-aminobutyrate transaminase. In one embodiment, the transgenic plant or the transgenic organism, wherein said one or more cytochrome P450 enzyme(s) and/or said γ-aminobutyrate transaminase is selected from among SEQ ID NOs:1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, or 26. In an embodiment the transgenic plant or the transgenic organism is selected from among a species of Brachypodium, a species of Setaria, a species of Populus, tobacco, corn, rice, soybean, cassava, canola (rapeseed), wheat, peanut, palm, coconut, safflower, sesame, cottonseed, sunflower, flax, olive, safflower, sugarcane, castor bean, switchgrass, Miscanthus, Camelina and Jatropha. In another embodiment the transgenic plant or the transgenic organism, wherein said heterologous nucleotide sequence is codon-optimized for expression in said transgenic plant. In yet another embodiment, the transgenic plant or the transgenic organism, wherein said heterologous nucleotide sequence is expressed in a tissue or organ selected from among an inflorescence, a flower, a sepal, a petal, a pistil, a stigma, a style, an ovary, an ovule, an embryo, a receptacle, a seed, a fruit, a stamen, a filament, an anther, a male or female gametophyte, a pollen grain, a meristem, a terminal bud, an axillary bud, a leaf, a stem, a root, a tuberous root, a rhizome, a tuber, a stolon, a corm, a bulb, an offset, a cell of said plant in culture, a tissue of said plant in culture, an organ of said plant in culture, and a callus.

[0080] In another embodiment, the invention relates to a method of making the transgenic plant or the transgenic organism, comprising the steps of: (i) inserting into the genome of a plant cell or an organism cell a heterologous nucleotide sequence comprising, operably linked for expression: (a) a promoter sequence; (b) at least one heterologous nucleotide sequence coding for a cytochrome P450 enzyme, and/or a γ-aminobutyrate transaminase; (ii) obtaining a transformed plant cell or a transformed organism cell; and (iii) regenerating from said transformed plant cell or said transformed organism cell a genetically transformed plant or a genetically transformed organism, wherein said genetically transformed plant or said genetically transformed organism produces cyclopamine and/or verazine-derived metabolite. In another embodiment the invention relates to a transgenic plant or a transformed organism made by the method.

[0081] In a further embodiment, the invention relates to a method of obtaining or producing cyclopamine and/or verazine-derived metabolite, comprising recovering cyclopamine from a transgenic plant or a transgenic organism. In yet another embodiment the invention contemplates cyclopamine and/or verazine-derived metabolite made by the method. In yet another embodiment, the invention relates to a method of preparing a cyclopamine and/or verazine-derived metabolite containing pharmaceutical composition, comprising formulating cylcopamine and/or verazine-derived metabolite as a pharmaceutical composition comprising a pharmaceutically acceptable carrier, dilient, or excipient, wherein said cyclopamine and/or verazine-derived metabolite is recovered from a transgenic plant. In a further emobodiment, the pharmaceutical composition, wherein said transgenic plant or said transgenic organism is made by the method.

[0082] In another embodiment, the invention relates to a pharmaceutical composition comprising cyclopamine and/or verazine-derived metabolite, wherein said cylcopamine and/or verazine-derived metabolite is obtained by growing a plant or an organism, and recovering cyclopamine and/or verazine-derived metabolite from said plant or said organism. Cyclopamine and/or verazine-derived metabolite for use in human therapy, wherein said cyclopamine and/or verazine-derived metabolite is recovered from a transgenic plant or an organism; and/or wherein said transgenic plant or transgenic organism is made by the method; and/or wherein said cyclopamine and/or verazine-derived metabolite is produced by the method. Use of cyclopamine and/or verazine-derived metabolite in human and/or animal therapy, wherein said cyclopamine and/or verazine-derived metabolite is recovered from a transgenic plant or a transgenic organism; and/or wherein said transgenic plant or said transgenic organism is made by the method; and/or wherein said cyclopamine and/or verazine-derived metabolite is produced by the method. Use of cyclopamine and/or verazine-derived metabolite for the preparation of a medicament for the treatment of cancer, wherein said cyclopamine and/or verazine-derived metabolite is recovered from a transgenic plant or a transgenic organism; and/or wherein said transgenic plant is made by the method; and/or wherein said cyclopamine and/or verazine-derived metabolite is produced by the method.

III. Biochemical Pathway Elucidation

[0083] The chemical diversity of plant natural products has provided humans with a variety of intriguing structures and biological activities. Due to these biological activities, 25% of medicines today are either derived directly from plants or are structural modifications of plant

natural products. An understanding of how these molecules are formed would serve a dual role to enable a study of the *in planta* function, as well as development of a synthetic biology production platform.

[0084] Natural products typically do not accumulate to high levels in the plant. If the source plant for a novel drug is not amenable to cultivation, drug development can be precluded. Engineering of a natural product biosynthetic pathway into an easily cultivated host plant can result in a sustainable supply of a drug. The first obstacle to this approach, however, is knowledge of the underlying biosynthetic genes.

[0085] Absent knowledge concerning the underlying genes or enzymes involved in a biosynthetic pathway, the candidate gene selection process requires extensive enzymatic and bioinformatic analysis concerning protein prediction, annotation, and phylogenetic relationships. Initial transciptome sequencing of different *Veratrum californicum* tissue cDNAs led to an extensive list of more than 56,000 expressed genes, none of which had been previously characterized. Moreover, many of the intermediates and metabolites are transitory and structurally unusual and/or complex; requiring multiple qualitative and quantitative detection methods, which add additional levels of difficulty to identification of genes within the biosynthetic pathway. In addition, further complicating biosynthetic pathway analysis, several of the enzymes identified herein do not exhibit single-substrate specificity, *i.e.*, one enzyme may catalyze multiple reactions. Thus, the inventors performed detailed analyses to distinguish metabolites of interest from intermediates and/or side products to elucidate the order and steps involved in the biosynthesis of cyclopamine.

[0086] Elucidation of biochemical pathways is of importance to understanding an organism on the molecular level. From a biotechnological perspective, knowledge of underlying genes of enzymes involved in biochemical synthesis is also required for suppressing, modifying, or even refactoring entire pathways on a synthetic biology platform. Protein purification and mining of cDNA libraries often required decades to elucidate complete pathways.

[0087] Biochemical pathway elucidation in non-model systems has often taken decades to complete. A prominent example is the well-known plant natural product morphine produced by the opium poppy *Papaver somniferum*. Though discovered in the early 1800's, the biosynthetic pathway is still not completely elucidated at the gene level. Much of the enzyme discovery work was accomplished by antibody screening, protein purification, amino acid sequencing, and subsequent cloning based upon those sequences. Genes encoding only 6 of

the 8 enzymes committed to the biosynthesis of morphine have been isolated and characterized from the 1990's to the present, i.e., several decades of work to uncover fewer than 8 genes.

[0088] Next-gen sequencing technology enables revolutionary new approaches to biochemical pathway discovery in the non-model system. Nucleotide sequence data acquisition is achieved at a previously unparalleled rate; however, bioinformatic interrogation of these large data sets remains a challenge. A combination of bioinformatics and next-gen sequencing has the potential to shorten natural product pathway discovery in non-model systems from several decades to several years. Methods employing next-gen sequencing technology are currently being tested in this respect.

[0089] Presented herein is a broadly applicable biosynthetic gene discovery method that is based on correlating plant metabolite accumulation with RNAseq gene expression data. As proof-of-concept of this method, the biosynthetic pathway to the complex steroid alkaloid cyclopamine was chosen.

[0090] To identify genes in this pathway, the inventors interrogated a *V. californicum* RNAseq dataset using a cyclopamine accumulation profile as the predefined model for gene expression with the pattern-matching algorithm Haystack. The inventors have identified and refactored in *Spodoptera frugiperda* Sf9 cells four *V. californicum* enzymes that catalyze the first six steps from cholesterol in the biosynthesis of the steroid alkaloid cyclopamine. The pathway refactoring method developed eliminates the need to synthesize and purify biosynthetic intermediates for validation of pathway enzyme activity.

[0091] Three of the newly discovered enzymes, i.e., cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, and 22-hydroxy-26-aminocholesterol 22-oxidase, are cytochromes P450. The fourth enzyme is a γ-aminobutyrate transaminase that catalyzes the transfer of nitrogen to 22-hydroxycholesterol-26-al. Enzymatic activity was confirmed by refactoring the plant pathway in *Spodoptera frugiperda* Sf9 cells. Structure elucidation of the enzymatic products was achieved by GC-MS, LC-MS/MS and NMR spectroscopy.

IV. Examples

[0092] The following examples are provided to illustrate various aspects of the present disclosure, and should not be construed as limiting the disclosure only to these particularly disclosed embodiments.

[0093] The materials and methods employed in the examples below are for illustrative purposes only, and are not intended to limit the practice of the present embodiments thereto. Any materials and methods similar or equivalent to those described herein as would be apparent to one of ordinary skill in the art can be used in the practice or testing of the present embodiments.

Example 1

RNA extraction

[0094] *V. californicum* plant material was obtained from wild populations in northern Utah. Tissue culture was initiated from wild collected seed and grown in the dark at 24°C on a combination of Linsmaier and Skoog vitamins and Murashige and Skoog media supplemented with 0.5 mg/l 1-naphthaleneacetic acid (Sigma). Refer to Table 6 for full media components. RNA extraction for each tissue (bulb, flower, leaf, fall rhizome, spring rhizome, root, green shoot, white shoot, and tissue culture samples) was performed as previously described (protocol 13). RNA quantity and integrity were evaluated with a NanoDrop 2000 (Thermo Scientific) and a Bioanalyzer 2100 (Agilent Technologies) prior to cDNA library preparation.

[0095] Table 6: LS/MS Rooting Media. LS/MS media was prepared with the following concentrations and brought to a final pH of 5.75.

Macronutrients	Supplier	Final Concentration (mg/l)
NH_4NO_3	Phytotechnology laboratories	1650
KNO_3	Sigma	1900
$MgSO_4$ x 7H2O	Sigma	370
KH_2PO_4	Sigma	170
$CaCl_2$ x2 H_2O	Sigma	440

Iron		
Na_2EDTA x 2 H_2O	Sigma	37.3
$FeSO_4$ x 7 H_2O	Sigma	27.8
Micronutrients		
H_3BO_3	Sigma	6.2
$MnSO_4$ x H_2O	Phytotechnology laboratories	16.9
$ZnSO_4$ x $7H_2O$	Sigma	8.6
KI	Sigma	0.83
Na_2MoO_4 x 2 H_2O	Phytotechnology laboratories	0.25
$CuSO_4$ x 5 H_2O	Sigma	0.025
$CoCl_2$ x $6H_2O$	Sigma	0.025
Vitamines		
Thiamine HCl	Sigma	0.1
Nicotinic Acid	Sigma	0.5
Pyroxidine HCl	Sigma	0.5
myo-inositol	Sigma	100
Other		
Sucrose	Phytotechnology laboratories	30000
1-naphthaleneacetic acid	Phytotechnology laboratories	0.5
Gelzan	Phytotechnology laboratories	3000

Liquid Chromatography Mass Spectrometry (LC-MS/MS) Method

[0096] Liquid chromatographic separation was achieved with 10 µl injections on a LC-20AD (Shimadzu) LC system coupled to a 4000 QTRAP (AB Sciex Instruments) for MS/MS analysis. Separation was achieved using a Phenomenex Gemini C-18 NX column (150 X 2.00 mm, 5 µm) with a flow rate of 0.5 ml/min and the following gradient program [solvent

A (0.05% formic acid/0.04% ammonium hydroxide (25%) v/v in H_2O; solvent B (0.05% formic acid/0.04% ammonium hydroxide (25%) v/v in 90% acetonitrile]: Solvent B was held at 20% for 2 min, then 2-11 min 20-30% B, 11 – 18 min 30-100% B, 18-22 min 100% B, 22-23 min 100-20% B, and held at 20% B for an additional 5 minutes. Program parameters included a TurboIonSpray ionization source temperature of 500°C and low resolution for Q1 and Q3 done with MRM (Multiple Reaction Monitoring) scans in the positive ion mode. Specific ion fragments and parameters can be found in Table 7. In conjunction, EMS (Enhanced MS) scan with a mass range of 380 to 425 m/z, and EPI (Enhanced Product Ion) scans for 398, 417, and 418 m/z were included in the method. Compound identification was determined by comparison of retention time and fragmentation pattern to the authentic standard cyclopamine (where applicable). Quantitation was performed by plotting peak area versus pmol of standard using Analyst 1.5 (Applied Biosystems).

[0097] Table 7: Q-TRAP 4000 Method Parameters

	Q1 Mass	Q3 Mass	Dwell (msec)	DP	CE
Veratramine	410	392	100	120	40
	410	295	100	120	40
Cyclopamine	412	321	100	120	40
	412	394	100	120	40
Muldamine	458	398	100	100	47
	458	253	100	100	47
22-Keto 26-hydroxycholesterol	417	271	100	70	30
	417	253	100	70	30
22-hydroxy-26-amino-cholesterol	418	400	100	70	30
	418	382	100	70	30
Verazine	398	253	100	70	60
	398	159	100	70	60

Gas Chromatography Mass Spectrometry (GC-MS) Method

[0098] Samples were first extracted with either hexane:isopropanol 3:2 followed by hexane only or ethyl acetate. Dried extracts were derivatized with 40 µl Sylon HTP (Sigma) for 1 hour at 90°C prior to injection with a 7683B autosampler onto a 7890A gas chromatograph coupled to a 5975C mass spectrometer inert XL MSD with triple-axis detector (Agilent Technologies). Both full scan and SIM methods were run in the splitless mode with 1 µl injection volume and a flow rate of 1 ml/min with helium as the carrier gas. Separation was performed on a Zebron ZB-5MSi column with guardian 5M (30 m x 0.25 mm x 0.25 µm) with 5% Polysilarylene - 95% Polydimethylsiloxane copolymer composition and 106 relative voltage. The initial temperature of 240°C was held for 5 minutes and increased to 300°C at a rate of 10°C/min and held for 25 minutes. The full scan method measured mass from 50 to 800 amu and ions detected in the SIM mode included: 99.1, 129, 165, 171, 173.1, 187, 261, 314.1, 329.3, 330, 370, 382.3, 417.4, 456.4, 458, 460, 470, 472.3, 486, 546, 560, and 634.

Metabolite extraction and quantitation by LC-MS/MS

[0099] Quantitation of cyclopamine in extracts from *V. californicum* was performed by LC-MS/MS. Extracts were prepared by grinding frozen plant tissue in liquid nitrogen followed by 5 minutes of vortexing in 70% ethanol added in a 200 µl to 100 mg w/v ratio. Samples were subject to centrifugation for 10 minutes (14,000 X g) at room temperature and the supernatant filtered through a 0.2 µm PTFE membrane (Millipore) prior to injection. Extracts were diluted 10-10,000 fold with 70% ethanol, depending on alkaloid content, prior to LC-MS/MS analysis (see LC-MS/MS method above).

Transcriptome assembly and retrieval of expression data

[0100] cDNA library construction, Illumina paired-end sequencing, and *de novo* transcriptome assembly were performed at the National Center for Genome Resources (Santa Fe, New Mexico). For the transcriptome assembly, 54 bp paired-end Illumina reads for each tissue were first examined for gross abnormalities and poor sequence quality and trimmed with the FASTX Toolkit. Subsequently short contig assembly was performed using the de Bruijn graph-based assembler ABySS several times with varying kmer lengths to generate 20 sets of synthetic ESTs with lengths between 100 – 500 base pairs. ABySS scaffolder was used to scaffold the synthetic ESTs and GapCloser from SOAPdenovo to close the NNN gap spacers. Lastly, the assembly was completed by combining the obtained scaffolds using Mira in the EST assembly mode. Post processing included translational predictions for each contig

using ESTSCAN and determination of expression data by alignment analysis of the trimmed reads to the assembled contigs using BWA.

[0101] To further enable comparison of gene expression between various tissues, the number of reads aligned to each contig was normalized by dividing by the total number of reads from the respective tissue sample. Functional annotations to each predicted protein sequence were obtained using Pfam, Superfamily, and Uniprot.

Haystack modeling

[0102] Identification of genes whose expression pattern correlated with accumulation of cyclopamine was determined using the Haystack program. The LC-MS/MS cyclopamine quantitation data for the different *V. californicum* tissues was used to formulate a model based upon the ratio of biosynthetic tissues. 95 % of the total cyclopamine was found in the subterranean tissues (root, bulb, and rhizome) whereas 5 % was found above ground (leaf, stem, and flower). For the input model, each subterranean tissue was given a value of 20 and all above ground tissues including the tissue culture samples was designated 1. Parameters for Haystack were as follows: correlation cut off = 0.7 fold change = 2, p-value = 0.05 and background = 1. Due to the large data input, Haystack analysis was performed on a UNIX server in-house as opposed to the version available online. Annotation data was then merged with the gene outputs from each of the models. Subsequent alignments and phylogenetic analysis were performed using Muscle algorithm and Mega v6.06.

Construction of viral expression vectors

[0103] Candidate contigs obtained from Haystack analysis were subjected to BLAST searches (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and global alignments to homologous, experimentally characterized gene sequences with the CLC Main Workbench 6.8, for prediction of the open reading frame. Where the reading frame appeared incomplete, Rapid Amplification of cDNA Ends (RACE) was used to obtain the complete coding sequence. *V. californicum* cDNA was prepared from root RNA extracts using M-MLV Reverse Transcriptase (Invitrogen) according to manufacturer's instructions. All primer sequences and PCR programs can be found in the Sequence Listing (SEQ ID NO:27-48) and Table 8, respectively.

[0104] Table 8: PCR Parameters

VC2646 for TOPO cloning, VC2646 for pVL1392 cloning, VC12709 for pVL1392 cloning, VC13284 TOPO cloning	98°C for 30 sec, then 35 cycles of 98°C for 10 sec, 60°C for 30 sec, 72°C for 1 min 30 sec, and a final 10 min extension at 72°C
VC12709 5' RACE	98°C for 30 sec, then 35 cycles of 98°C for 10 sec, 65°C for 30 sec, 72°C for 1 min and a final 10 min extension at 72°C
VC13284 for pVL1392 cloning	98°C for 30 sec, then 35 cycles of 98°C for 10 sec, 63°C for 30 sec, 72°C for 1 min 25 sec, and a final 10 min extension at 72°C
VC12084 for pVL1392 cloning	98°C for 30 sec, then 35 cycles of 98°C for 10 sec, 60°C for 30 sec, 72°C for 1 min, and a final 10 min extension at 72°C
Tomato GABAT and VC674 for pVL1392 cloning	98°C for 30 sec, then 35 cycles of 98°C for 10 sec, 62°C for 30 sec, 72°C for 45 sec, and a final 10 min extension at 72°C
Semi-Quantitative RT-PCR	95°C for 2 min, then 26 cycles of 95°C for 30 sec, 50°C for 30 sec, 72°C for 15 sec

[0105] The cDNAs encoding cholesterol 22-hydroxylase (accession numbers KJ869252, KJ869253), 22-hydroxy-26-aminocholesterol 22-oxidase (accession numbers KJ869258-KJ869261), 22-hydroxycholesterol-26-al transaminase (accession numbers KJ869262-KJ869264) and γ-aminobutyrate (GABA) transaminase 2 (accession number KJ869265) were determined to be full length. Cholesterol 22-hydroxylase and 22-hydroxy-26-aminocholesterol 22-oxidase were amplified by Polymerase Chain Reaction (PCR) from cDNA with Phusion DNA polymerase (New England Biolabs) using primers 1-4 and 7-8, respectively, and initially ligated into the pCR-Blunt II-TOPO vector (Invitrogen). Two rounds of amplification were required for cholesterol 22-hydroxylase by nested PCR. Subsequently, cholesterol 22-hydroxylase was amplified from pCR-Blunt II-TOPO with primers 5 and 6 introducing NotI/BamHI restriction sites into the PCR products at the 5' and 3' ends of the open reading frame. The amplified product and pVL1392 Baculovirus transfer vector (BD Biosciences) were digested with NotI/BamHI and ligated together using Rapid Ligase (Promega). Ligated constructs were transformed into *E. coli* DH5α competent cells.

22-Hydroxy-26-aminocholesterol 22-oxidase was amplified with primers 9 and 10, introducing PstI/XbaI restriction sites at the 5' and 3' end of the open reading frame. The amplified product, along with pVL1392, was digested with PstI/XbaI and subject to ligation and transformation.

[0106] 22-Hydroxycholesterol-26-al transaminase and GABA transaminase 2 were directly amplified from cDNA using primers 11, 12 incorporating BglII/EcoRI restriction sites at the 5' and 3' end of the open reading frame and 21, 22, incorporating PstI/XbaI restriction sites at the 5' and 3' end of the open reading frame, respectively. 22-Hydroxycholesterol-26-al transaminase and pVL1392 were subject to restriction digest with BglII/EcoRI preceding ligation and transformation. GABA transaminase 2 was digested with XbaI/PstI, preceding ligation and transformation.

[0107] RACE was required to determine the 5' sequence of 22-hydroxycholesterol 26-hydroxylase/oxidase gene (accession numbers KJ869254-KJ869257). RACE ready cDNA was prepared using the GeneRacer Kit (Invitrogen) according to manufacturer's instructions using *V. californicum* root RNA. Primers 13 and 15 were used for PCR (round 1), followed by amplification using primers 14 and 16 (round 2). Resulting RACE fragments were cloned into PCR-Blunt II-TOPO. The full-length gene was directly amplified from *V. californicum* root cDNA with primers 17 and 18, incorporating BglII/EcoRI restriction sites at the 5' and 3' end of the open reading frame. The amplified product was digested with Bglll/EcoRI and ligated into pVL1392 digested with the same enzymes. Each characterized *V. californicum* contig and subsequent enzyme designation can be found in Table 1.

[0108] Table 1. Enzyme assignments

Transcriptome derived Contig designations	**CYP designation**	**Accession numbers**	**Assigned name based on function**
>medp_verca-20110208|2646	CYP90B27v1	KJ869252	Cholesterol 22-hydroxylase
	CYP90B27v2	KJ869253	Cholesterol 22-hydroxylase
>medp_verca-20110208|12709	CYP94N1v1	KJ869254	22-Hydroxycholesterol 26-hydroxylase/oxidase
	CYP94N1v2	KJ869255	22-Hydroxycholesterol

			26-hydroxylase/oxidase
	CYP94N2v1	KJ869256	22-Hydroxycholesterol 26-hydroxylase/oxidase
	CYP94N2v2	KJ869257	22-Hydroxycholesterol 26-hydroxylase/oxidase
>medp_verca-20110208|12084	N/A	KJ869262	22-Hydroxycholesterol-26-al transaminase
	N/A	KJ869263	22-Hydroxycholesterol-26-al transaminase
	N/A	KJ869264	22-Hydroxycholesterol-26-al transaminase
>medp_verca-20110208|13284	CYP90G1v1	KJ869258	22-Hydroxy-26-aminocholesterol 22-oxidase
	CYP90G1v2	KJ869261	22-Hydroxy-26-aminocholesterol 22-oxidase
	CYP90G1v3	KJ869260	22-Hydroxy-26-aminocholesterol 22-oxidase
	CYP90G2	KJ869259	22-Hydroxy-26-aminocholesterol 22-oxidase

[0109] The cDNA encoding GABA transaminase isozyme 2 from *Solanum lycopersicum* (tomato) implicated in steroid alkaloid biosynthesis (accession number AY240230) was isolated from *S. lycopersicum* using the Qiagen RNA-easy kit for RNA extraction followed by cDNA synthesis as described above. *S. lycopersicum* GABA transaminase isozyme 2 was amplified by PCR using Primers 19 and 20, incorporating PstI/XbaI sites at the 5' and 3' end of the open reading frame. The amplified product and pVL1392 were subject to restriction digest with PstI/XbaI and ligated together, preceding transformation.

Virus co-transfection, amplification, and protein production

[0110] Each pVL1392 expression construct was independently co-transfected with the Baculogold Linearized Baculovirus (BD Biosciences) into *S. frugiperda* Sf9 cells according to manufacturer's instructions. Sf9 cells were maintained as previously described. Virus

amplification and protein production proceeded as previously described. Each cytochrome P450 virus construct was co-expressed with *Eschscholzia californica* cytochrome P450 reductase (CPR) in *S. frugiperda* Sf9 cells. *S. frugiperda* Sf9 cell cultures were also infected with several constructs in parallel. Combinations of each cytochrome P450 can be found in Table 2. In addition, *V. californicum* 22-hydroxycholesterol-26-al transaminase was produced by single infection. Equal volumes for each virus were used in the multiple infections and adjusted to a total viral volume of 2.5 ml.

[0111] Table 2. Viral combination for in vivo production of metabolites in *Sf9* cells

Combination	Viruses
Combination 1	Cholesterol 22-hydroxylase, CPR
Combination 2	22-Hydroxycholesterol 26-hydroxylase/oxidase, CPR
Combination 3	22-Hydroxy-26-aminocholesterol 22-oxidase, CPR
Combination 4	Cholesterol 22-hydroxylase, 22-Hydroxycholesterol 26-hydroxylase/oxidase, CPR
Combination 5	Cholesterol 22-hydroxylase, 22-Hydroxy-26-aminocholesterol 22-oxidase, CPR
Combination 6	Cholesterol 22-hydroxylase, 22-Hydroxycholesterol 26-hydroxylase/oxidase, 22-Hydroxy-26-aminocholesterol 22-oxidase, CPR
Combination 7	Cholesterol 22-hydroxylase, 22-Hydroxycholesterol 26-hydroxylase/oxidase, 22-Hydroxy-26-aminocholesterol 22-oxidase, 22-Hydroxycholesterol-26-al transaminase, CPR
Combination 8	Cholesterol 22-hydroxylase, 22-Hydroxycholesterol 26-hydroxylase/oxidase, 22-Hydroxy-26-aminocholesterol 22-oxidase, γ-aminobutyrate transaminase 2, CPR
Combination 9	Cholesterol 22-hydroxylase, 22-Hydroxycholesterol 26-hydroxylase/oxidase, 22-Hydroxy-26-aminocholesterol 22-oxidase, *S. lycopersicum* GABA transaminase isozyme 2, CPR
Combination 10	Cholesterol 22-hydroxylase, 22-Hydroxycholesterol 26-hydroxylase/oxidase, 22-Hydroxycholesterol-26-al transaminase, CPR

Extraction of multiple infections for Sf9 *in vivo* product production

[0112] Baculovirus infections were carried out and insect cells were collected as stated above and used for production of each enzymatic product in *S. frugiperda* Sf9 cells. 1 ml each of *S. frugiperda* Sf9 cells expressing the various combinations of virus were extracted with 2 volumes of ethyl acetate by vortexing (1 min), centrifugation (16,000 x g; 2 min), and were taken to dryness under N_2. Samples were either derivatized with 40 µl of Sylon HTP and injected onto the GC-MS with the method stated above or re-suspended in 50 µl of 80% Methanol and analyzed by LC/MS-MS with the method stated above.

Enzyme assays

[0113] Each cytochrome P450 co-expressed with CPR in *S. frugiperda* Sf9 cells was subjected to individual enzyme assays with the compounds found in Table 3 to determine functionality. Compounds were prepared to 1 mM stock solutions of 100% DMSO and diluted with H_2O.

[0114] Table 3. Substrate testing for cytochrome P450 enzymes co-expressed with CPR. Substrate testing for cytochrome P450 enzymes co-expressed with CPR. Production of a detectable product is indicated by a (+). CYP90B27 refers to cholesterol 22-hydroxylase, CYP90G1 refers to 22-hydroxy-26-aminocholesterol 22-oxidase, and CYP94N1 refers to 22-hydroxycholesterol 26-hydroxylase/oxidase.

	CYP90B27	CYP90G1	CYP94N1
cholesterol	+	-	-
22(R)-hydroxycholesterol	+	+	+
22(S)-hydroxycholesterol	-	-	-
26-hydroxycholesterol	+	-	N/A
22,26-dihydroxycholesterol	N/A	+	N/A
22-keto-cholesterol	N/A	N/A	-
4β-hydroxycholesterol	-	-	-
7β-hydroxycholesterol	+	-	-
24(S)-hydroxycholesterol	-	-	-
campesterol	-	N/A	N/A
β-sitosterol	-	N/A	N/A
stigmasterol	-	N/A	N/A

Production of a detectable product is indicated by a Yes. Abbreviations for substrates are used for formatting purposes and the full names are as follows from right to left: 22(*R*)-Hydroxycholesterol, 22(*S*)-Hydroxycholesterol, 26-Hydroxycholesterol, 22, 26-Dihydroxycholesterol, 22-Keto cholesterol, 4β-Hydroxycholesterol, 7β-Hydroxycholesterol, and 24 (*S*)-Hydroxycholesterol.

[0115] Standards were obtained from Sigma Aldrich, Research Plus, and Avanti. For GC-MS analysis, 5 individual assays per substrate were pooled after incubation at 30°C for 2 hours; one assay produced sufficient product for analysis by LC-MS/MS. Assay conditions were as follows: 80 µl *S. frugiperda* Sf9 cell suspension (obtained by re-suspension of 50 ml viral infected culture pellet in 3.5 ml of 100 mM tricine pH 7.4/ 5 mM thioglycolic acid), 60 mM potassium phosphate buffer pH 8, 1.25 mM NADPH, 7.5 µM substrate, and H_2O in a total volume of 200 µl. Controls were performed with no enzyme and *S. frugiperda* Sf9 cells expressing an unrelated cytochrome P450, or CPR-only, for each assay.

[0116] The 22-hydroxycholesterol-26-al transaminase enzyme assay contained 55 µl *S. frugiperda* Sf9 cell suspension infected with cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxy-26-aminocholesterol 22-oxidase and CPR modified baculoviruses (to provide 22-hydroxycholesterol-26-al substrate), 40 µl *S. frugiperda* Sf9 cells expressing 22-hydroxycholesterol-26-al transaminase, 60 mM potassium phosphate buffer pH 8, 1.5 mM DTT, 100 µM pyridoxal-5-phosphate, 16 mM GABA, 500 µM NADPH, and water to a total volume of 200 µl. Assay mixes lacking either enzyme or GABA, and control cytochrome P450 assays were run in parallel and each was allowed to proceed for 2 hours at 30°C. Samples were extracted twice with 400 µl ethyl acetate. Samples were then dried under N_2, re-suspended in 50 µl 80% methanol, and injected onto LC-MS/MS with conditions described above. All enzyme assays utilized crude *S. frugiperda* Sf9 protein extracts that contain endogenous metabolites, including cholesterol.

Assays to clarify order of enzymatic transformations

[0117] Assay for GC-MS: Cytochrome P450 enzyme assay conditions were identical to those stated above using *S. frugiperda* Sf9 cell suspensions with the following modifications. First, 12 assays each containing 22-hydroxy-26-aminocholesterol 22-oxidase + CPR, 22-hydroxycholesterol 26-hydroxylase/oxidase + CPR, or control cytochrome P450 + CPR and each with pure 22(*R*)-hydroxycholesterol were allowed to incubate overnight at 30°C. Like assays were pooled, extracted 3 times with 2 volumes ethyl acetate, dried under N_2, and re-suspended in 180 µl of 25% DMSO. Extracts containing the enzymatic product of the 22-hydroxycholesterol 26-hydroxylase/ oxidase + CPR and 22(*R*)-hydroxycholesterol were divided equally and used as substrate for 22-hydroxy-26-aminocholesterol 22-oxidase + CPR and control cytochrome P450 + CPR. Extracts containing the enzymatic product of 22-hydroxy-26-aminocholesterol 22-oxidase + CPR and 22(*R*)-hydroxycholesterol were divided

and used as substrate in 6 assays containing 22-hydroxycholesterol 26-hydroxylase/ oxidase + CPR and 6 assays containing control cytochrome P450 + CPR. Control P450 + CPR assay was run in parallel, treated identically and added to another control P450 assay. Refer to Figure 1A for an overview of the experiment. Assays were allowed to incubate for 20 min at 30°C then stopped by addition of 20 µl of 20% TCA with vortexing. Like assays were pooled, extracted, derivatized, and analyzed by GC-MS using the method stated above.

[0118] Assay for LC-MS/MS: All assays utilized crude *frugiperda* Sf9 cell suspensions. Enzyme assays started with a combination of cholesterol 22-hydroxylase + CPR and 22-hydroxycholesterol 26-hydroxylase/oxidase + CPR (8 individual reactions) in parallel to cholesterol 22-hydroxylase + CPR and 22-hydroxy-26-aminocholesterol 22-oxidase + CPR (8 reactions). Assays were extracted, and fed to 22-hydroxycholesterol 26-hydroxylase/oxidase + CPR, 22-hydroxy-26-aminocholesterol 22-oxidase + CPR, or 22-hydroxycholesterol-26-al transaminase for several possible enzyme combinations (4 reactions each). Like samples were pooled, extracted, and added to 2 reactions each with enzyme not yet utilized previously. Refer to Figure 1B for complete list of combinations. Samples were taken at each step post extraction for LC-MS/MS analysis and run with the method stated above.

Enzymatic product purification for NMR and High Resolution MS for structure elucidation

[0119] Large-scale 750 ml *S. frugiperda* Sf9 cultures were grown expressing viral combinations 5-7 (Table 2) of the *V. californicum* enzymes as previously described. Cells were collected after three days and re-suspended in 10 ml of 100 mM tricine pH 7.4/ 5 mM thioglycolic acid; then extracted 3 times with 2 volumes of hexane or ethyl acetate. The remaining aqueous supernatant was extracted once with 1 volume of hexane or ethyl acetate. Extracts for each infection were then pooled, dried under N_2, and re-suspended in 5 ml of absolute methanol.

[0120] The extracts were purified on a Waters HPLC system equipped with a 2707 autosampler, 1525 binary pump, 2998 photodiode array detector, and Waters Fraction Collector III. In some cases, samples were cleaned up by Solid Phase Extraction (SPE), before HPLC purification. For HPLC extracts were concentrated to 500 µl and then injected in 50 µl portions onto a Phenomenex Gemini C-18 NX column (150 X 2.00 mm, 5µm) with the same solvents used for LC-MS/MS as described above with the following binary

gradient: Solvent B was held at 20% for 2 min, then 2-11 min 20-30% B, 11-18 min 30-100% B, 18-30 min 100% B, 30-31 min 100-20% B, and held at 20% B for an additional 5 minutes. The flow rate was 0.5 ml/min; 0.5 ml fractions were collected. The resulting fractions were then analyzed by GC-MS or LC-MS/MS as described above, and selected samples were analyzed by NMR or by high resolution MS. NMR spectra were acquired in MeOD at 600 MHz on a BrukerAvance 600 MHz spectrometer equipped with a BrukerBioSpin TCI 1.7 mm MicroCryoProbe. Proton, gCOSY, ROESY, gHSQC, and gHMBC spectra were acquired; ^{13}C chemical shifts were obtained from the HSQC and HMBC spectra. Chemical shifts are reported with respect to the residual non-deuterated MeOD signal. Refer to Tables 9 and 10 for NMR designations for 22-keto-cholesterol and 22-keto-26-hydroxycholesterol, respectively. For high resolution MS, the sample was diluted 1:10 in 80% acetonitrile:water (LC-MS grade) containing 0.1% formic acid and infused into an LTQ-Orbitrap Velos Pro (Thermo-Fisher Scientific, San Jose, CA) using a Triversa Nanomate (Advion, Ithaca, NY). Data were collected in positive ion mode, detected in the Orbitrap at a nominal resolution setting of 60,000 at m/z 400. Precursors were determined with a wide SIM scan (m/z 385-430). Precursors were isolated in the ion-trap and transferred to the HCD cell for fragmentation at 35 NCE (m/z 418) and 50 NCE (m/z 398). Data were analyzed manually using the Qualbrowser application of Xcalibur (Thermo-Fisher Scientific, San Jose, CA).

[0121] Table 9: 22-Keto-cholesterol NMR in MeOD

position	^{13}C	^{1}H
1	38.3	1.09 [a] 1.88 (*dt*, 13.0, 3.5)
2	31.8	1.49 [a] 1.78 *m*
3	72.1	3.40 *m*
4	42.6	2.23 *m*
5	141.9	
6	122.1	5.34 *m*
7	32.7	1.55 [a] 1.98 [a]
8	36.8	
9	51.4	0.98 (*td*, 11.0, 5.0)
10	37.7	
11	22.0	1.50 [a] 1.55 [a]
12	40.8	1.31 [a] 2.01 [a]
13	43.4	
14	57.3	1.07 [a]
15	25.8	1.60 [a]
16	28.2	1.19 *m* 1.62 [a]
17	53.6	1.58 [a]
18	12.2	0.76 *s*
19	19.6	1.03 *s*
20	50.2	2.59 *m*
21	16.7	1.10 (*d*, 7.0)
22	217.8	
23	40.4	2.45 *m* 2.54 *m*
24	33.1	1.41 *m*
25	28.6	1.52 [a]
26	13.2	0.90 (*d*, 6.8)
27	22.5	0.90 (*d*, 6.8)

[0122] Table 10: 22-keto-26-hydroxycholesterol (SF 20797). NMR in MeOD

position	^{13}C	^{1}H
1	38.3	1.09 [a] 1.88 (*dt*, 13.6, 3.3)
2	31.9	1.49 [a] 1.79 *m*
3	72.1	3.39 [a]
4	42.7	
5	142.1	
6	122.1	5.34 (*br d*, 5.1)
7	32.7	1.50 [a] 1.97 [a]
8	32.5	1.45 [a]
9	51.3	0.98 (*td*, 11.2, 5.0)
10	37.4	
11	21.8	1.53 [a] 1.57 [a]
12	40.6	1.30 [a] 2.01 [a]
13	43.2	
14	57.1	1.07 [a]
15	25.4	1.15 [a] 1.63 [a]
16	28.1	1.21 [a] 1.63 [a]
17	53.3	1.60 [a]
18	12.0	0.76 *s*
19	19.6	1.03 *s*
20	50.2	2.60 [a]
21	16.7	1.11 (*d*, 7.0)
22	217.0	
23	40.1	2.46 (*ddd*, 17.6, 9.2, 5.9) 2.62 [a]
24	27.4	1.31 [a] 1.67 [a]
25	35.9	1.56 [a]
26	16.6	0.91 (*d*, 6.6)
27	67.7	3.35 (*dd*, 10.6, 6.2) 3.40 [a]

Dimedone aldehyde trapping

[0123] Enzyme assays containing 22-hydroxycholesterol 26-hydroxylase/oxidase and 22(*R*)-hydroxycholesterol as substrate, or cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase utilizing endogenous cholesterol in *S. frugiperda* Sf9 cells as substrate, or cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase and 22-hydroxycholesterol-26-al transaminase, also utilizing endogenous cholesterol in *S. frugiperda* Sf9 cells as substrate with either 80 µl 10 mg/ml dimedone in 10% DMSO or 80 µl 10%

DMSO were incubated overnight at 30°C. Assays were extracted twice with 2 volumes ethyl acetate and analyzed by LC-MS/MS. All cytochrome P450 enzymes were co-expressed with CPR.

Sodium borohydride reduction

[0124] 2 ml *S. frugiperda* Sf9 cells expressing cholesterol 22-hydroxylase + 22-hydroxycholesterol 26-hydroxylase/oxidase + 22-hydroxycholesterol-26-al transaminase + 22-hydroxy-26-aminocholesterol 22-oxidase + CPR were extracted twice with equal volume ethyl acetate. Extracts were divided equally, dried under N_2, and re-suspended in 50 µl 80% methanol each. One sample was treated with 50 µl 1 M $NaBH_4$ in 1 M NaOH for 15 minutes. 100 µl H_2O were added to both samples, and each extracted twice with equal volumes of chloroform. Samples were dried under N_2, re-suspended in 50 µl 80% methanol and analyzed by LC-MS/MS as described above. *S. frugiperda* Sf9 cells expressing CPR only were run in parallel as control.

Semi-Quantitative RT-PCR

[0125] Semi-quantitative RT-PCR was performed on cDNA prepared from each *V. californicum* tissue using Taq DNA polymerase (New England Biolabs). Cycle parameters can be found in Table 8; primers specific to each gene can be found in the Sequence Listing (SEQ ID NO:27-48). Arabidopsis Protein Phosphatase 2A SubunitA2 (PP2AA2) cDNA sequence was used to BLAST the *V. californicum* transcriptome to find a suitable homolog to be used as a housekeeping gene for normalization. Resulting products were run on a 2% agarose gel and band intensity was quantitated with the image processing and analysis software Image J.

SDS-PAGE

[0126] SDS-PAGE was performed for each functional gene to verify recombinant protein production. 10% Mini-PROTEAN TGX (Biorad) precast gels were used on a Mini-PROTEAN Tetra Cell (Biorad). 1 µl *S. frugiperda* Sf9 cell suspension co-infected with *V. californicum* cytochrome P450 and CPR was loaded onto the gel alongside a CPR only control and pure BSA (Fisher Scientific). Protein bands were visualized by coomassie blue staining using commassie brilliant blue R-250 (Amresco).

Results and Discussion

RNA-Seq and *de novo* transcriptome assembly

[0127] Multiplex paired-end sequencing of *V. californicum* cDNA produced from bulb, flower, leaf, fall rhizome, spring rhizome, root, green shoot, white shoot, and tissue culture samples on two 2 x 50 bp Hi-Seq channels resulted in 41,106,915 bases of an average read count of 2,520. The raw reads in the HiSeq datasets were analyzed and filtered for artifacts/contaminants. The reads were 5′ and 3′ quality trimmed using a FRED score of 15 to eliminate noisy reads. The *de novo* short read assembly was produced with multiple runs of de Bruijn assembler (kmer sweep). Native Abyss scaffolding and gap closing was performed to produce collections of synthetic EST scaffolds. These scaffolds were merged and assembled with Mira, and any remaining redundancy was removed to produce a final contig set. Post-processing included protein prediction as FASTA, protein product motif annotation as GFF3, and post-hoc alignment of cleaned read data to contigs. The results of the dataset processing and assembly produced 56,994 contigs. The depth of the transcriptome sequencing was sufficient to utilize mapped read-counts as a metric of relative gene expression. The average contig sequence length indicates high quality assembly and was sufficient for downstream sequence alignment and phylogenetic gene tree estimation.

Transcriptome dataset interrogation

[0128] Predicted peptide sequences were submitted to Pfam, Uniprot, and Superfamily in addition to BLAST search at NCBI to provide an annotation to each translated contig. Expression data for each contig was normalized using total reads per organ type to serve as the dataset for Haystack. LC-MS/MS determination of the steroid alkaloid profile in the same *V. californicum* tissues used for RNA-Seq resulted in a pronounced accumulation of cyclopamine in rhizome, fall rhizome being the highest, followed by root and bulb (Figure 2). The accumulation of highest quantities of cyclopamine in subterranean organs suggests that biosynthesis occurs in underground organs of the plant. Transport of metabolites in plants has been demonstrated (i.e. nicotine in tobacco and cyanogenic glucosides in cassava), but secondary metabolites most often are synthesized at or near their site of accumulation. Since little is known about cyclopamine biosynthesis in *Veratrum*, it was initially hypothesized that underground tissues (rhizome, root, and bulb) are biosynthetic for cyclopamine.

[0129] Because *ca.* 20 times more cyclopamine accumulates in subterranean organs compared to aerial organs, root, rhizomes, and bulb were given a value of 20 for the Haystack input. The above ground organs leaf, stem, flower, and tissue culture samples (derived from seed) were designated a value of 1 in order to create a generalized model based on biosynthesis. Haystack uses a model-based, pattern-matching algorithm to identify genes with expression patterns that fit a predefined input model (here cyclopamine accumulation). In our approach, the LC-MS/MS alkaloid data for *Veratrum* is the input *model* used to search the deep transcriptome experimental *dataset* of *Veratrum*. Haystack determines the correlation of the experimental dataset with each input model pattern and applies a series of statistical tests and *ad hoc* filters to identify genes of interest. Using a correlation cut off of 0.7, 3,219 genes were obtained that fit the 20:1 subterranean organ:aerial organ cyclopamine accumulation model.

[0130] In parallel to co-localization modeling, the protein-coding gene sequences in the *Veratrum* RNAseq transcriptome dataset were classified into putative gene families using PlantTribes 2.0. PlantTribes is based on the similarity-based clustering procedure TribeMCL, and incorporates the *Veratrum* protein sequences into existing plant tribe alignments and phylogenies. In addition to this tribe clustering approach, a complete minimal representative dataset from all available plant species of cytochromes P450 relevant to alkaloid biosynthesis was developed. Cytochromes P450 were chosen first due to the hypothesized number of oxidative transformations necessary to convert cholesterol into cyclopamine. Our experience in plant alkaloid biosynthesis has taught us that these types of transformations are typically catalyzed by cytochromes P450. The second choice of enzyme class would be 2-oxo-glutarate-dependent dioxygenases, should the cytochrome P450 dataset not yield positive results.

[0131] This dataset can be used to better define and cluster the tribes that are of most interest to the cyclopamine pathway. Multiple sequence alignment and phylogenetic tree estimation were done on these relevant tribes and gene families using the MAFFT alignment software and RAxML for maximum likelihood tree generation. In addition to *Veratrum*, the RNAseq transcriptome assembly sequences from *Colchicum autumnale* (autumn crocus) and *Narcissus* (daffodil) were included in the tribe clustering steps of the computational pipeline. Similar to *Veratrum*, these two species are also monocots. However, *Colchicum* and *Narcissus* do not produce cyclopamine, but instead make the unrelated alkaloids colchicine

and galanthamine, respectively. Therefore, *Colchicum* and *Narcissus* sequences helped identify tribe clusters that only contain *Veratrum* genes.

[0132] A series of selection criteria were established to score and sort the resulting clades. A given clade was scored on the percentage of clade members that significantly co-localized with cyclopamine (e.g. present in the Haystack output dataset). Clades missing significantly co-localized gene members were penalized. Clades containing genes that were *not* significantly co-localized with the alkaloid were penalized. Lastly, clades that contain genes from species that *do not* produce cyclopamine incurred a score penalty. Therefore, the *Colchicum* and *Narcissus* gene sequences served as controls in the clade-scoring portion of the computational pipeline. These criteria were combined to score and rank the clades that contain Haystack output gene members to identify the clade(s) with the highest likelihood of containing genes that function in the steroid alkaloid biosynthesis pathway. Candidate genes from clades with the highest scores were selected for downstream functional characterization (Table 4; Figure 10).

[0133] Table 4. Selected top-scoring cytochrome P450 candidate cDNAs for the enzymatic conversion of cholesterol to cyclopamine

Gene ID	Putative function
>medp_verca-20110208|2398	similar to CYP71D unknown function
>medp_verca-20110208|31930	similar to CYP71D unknown function
>medp_verca-20110208|10041	similar to CYP728 taxane 13a-hydroxylase
>medp_verca-20110208|13942	similar to CYP734 brassinolide C-26 hydroxylase
>medp_verca-20110208|13284	similar to CYP90B1 steroid C-22 hydroxylase
>medp_verca-20110208|18017	similar to CYP90B1 steroid C-22 hydroxylase
>medp_verca-20110208|18580	similar to CYP90B1 steroid C-22 hydroxylase
>medp_verca-20110208|2646	similar to CYP90B1 steroid C-22 hydroxylase
>medp_verca-20110208|32399	similar to CYP90B1 steroid C-22 hydroxylase
>medp_verca-20110208|12709	similar to CYP94D unknown function

[0134] Since a nitrogen atom must be introduced into the steroid skeleton to produce an alkaloid, aminotransferases fitting the model were included in the candidate gene list as well (Table 5).

[0135] Table 5. Selected top-scoring transaminases in the steroidal alkaloid biosynthetic pathway

Gene ID	Putative function
>medp_verca-20110208|12217	aminotransferase ACS10
>medp_verca-20110208|12084	gamma aminobutyrate transaminase 1, mitochondrial-like
>medp_verca-20110208|5285	1-aminocyclopropane-1-carboxylate synthase
>medp_verca-20110208|28717	aminotransferase ACS12-like
>medp_verca-20110208|15871	histidinol-phosphate aminotransferase, chloroplastic-like
>medp_verca-20110208|10159	cysteine desulfurase 1
>medp_verca-20110208|1461	methionine S-methyltransferase

[0136] Full-length candidate cDNAs were expressed using *S. frugiperda* Sf9 cells using a baculovirus-based expression vector due to the suitability of insect cells for producing functional post transcriptionally-modified, membrane-bound proteins, and for the ability to accommodate multiple-virus infections. *S. frugiperda* Sf9 cells provide a facile synthetic biology platform for the systematic refactoring of plant biosynthetic pathways.

Cholesterol 22-hydroxylase

[0137] The top-scoring candidate cDNAs resulting from interrogation of the *V. californicum* transcriptome dataset were systematically introduced, together with *E. californica* cytochrome P450 reductase (CPR), into *S. frugiperda* Sf9 insect cells, which were harvested as previously described and used in enzyme assays with cholesterol as substrate. Cholesterol was chosen as the initial precursor for study based upon existing knowledge of steroid alkaloid biosynthesis. Other related compounds were also tested in enzyme assays to determine enzyme specificity (Table 3). The contig designated VC2646, which annotated as a steroid C-22 hydroxylase, added a hydroxyl group to the 22-position of cholesterol exclusively in the *R* orientation (Figure 3; Figure 15A). One homolog of VC2646 was identified having 99.8% identity and performing the same enzymatic function. CYP

assignments for both homologs are CYP90B27v1 and CYP90B27v2. The inventors have designated this enzyme cholesterol 22-hydroxylase. *S. frugiperda* Sf9 cells expressing cholesterol 22-hydroxylase and the *E. californica* CPR demonstrated that this enzyme could produce the product *in vivo* during viral infection utilizing endogenous *S. frugiperda* cholesterol. Cholesterol 22-hydroxylase also hydroxylated 26-hydroxycholesterol and 7β-hydroxycholesterol, presumably in the 22-position. Cholesterol 22-hydroxylase oxidizes the hydroxyl group at the 22-position to a ketone, but only to a low degree (Figure 3). The identity of the enzymatic product of cholesterol 22-hydroxylase acting on cholesterol was confirmed by GC-MS comparison to 22(*R*)-hydroxycholesterol authentic standard. 22(*R*)- and 22(*S*)-hydroxycholesterol are chromatographically resolved by this GC-MS method.

22-Hydroxycholesterol 26-hydroxylase/oxidase

[0138] To identify the second enzyme in the pathway, a series of triple infections of *S. frugiperda* Sf9 cells were carried out that all contained cholesterol 22-hydroxylase and *E. californica* CPR, but varied the second enzyme. Candidates for the second enzyme were the remaining top-scoring candidate cDNAs resulting from interrogation of the *V. californicum* transcriptome dataset (minus the cholesterol 22-hydroxylase already identified). Contig VC12709 annotated as a fatty acid hydroxylase and was found to hydroxylate 22(*R*)-hydroxycholesterol at the C-26 position forming 22,26-dihydroxycholesterol (Figure 3). This enzyme also oxidizes the hydroxyl group at the 26 position creating a highly reactive 22-hydroxycholesterol-26-al (Figure 11A). Four homologs were discovered, with identities ranging from 93–99% and all possessing identical functionality. Hydroxylation of cholesterol by VC12709 was not detected (Figure 15B), so this enzyme was subsequently designated 22-hydroxycholesterol 26-hydroxylase/oxidase. CYP assignments for these homologs are CYP94N1v1, CYP94N1v2, CYP94N2v1, and CYP94N2v2. The identity of 22,26-dihydroxycholesterol produced by action of VC12709 on 22(*R*)-hydroxycholesterol was ultimately determined using the 22-hydroxylating activity of cholesterol 22-hydroxylase to produce 22,26–dihydroxycholesterol from pure 26-hydroxycholesterol and comparing the mass spectra of the two products.

22-Hydroxycholesterol-26-al transaminase

[0139] To identify the third enzyme in the pathway, a series of quadruple infections of insect cells were carried out that all contained cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, and *E. californica* CPR, but varied the third enzyme. Candidates for

the third enzyme were the remaining top-scoring candidate cDNAs resulting from interrogation of the *V. californicum* transcriptome dataset (minus the two enzymes already identified). A GABA transaminase designated VC12084 was shown to incorporate nitrogen into the 26-position of 22-hydroxycholesterol-26-al using GABA as an amino group donor to produce 22-hydroxy-26-aminocholesterol (Figure 4). Three homologs were detected, each with over 99% identity and all catalyzing the same reaction. The structure of 22-hydroxy-26-aminocholesterol was confirmed by high resolution MS (Figure 12). This enzyme was subsequently designated 22-hydroxycholesterol-26-al transaminase. In corroboration of our results, addition of the hydroxyl group followed by nitrogen addition to the 26-position is supported by early studies using *Veratrum grandiflorum* in which 22(*R*),26-dihydroxycholesterol was found to be a predominant sapogenin in budding *V. grandiflorum* extracts and surmised to be a precursor to the nitrogen-containing metabolite verazine, and recent studies in *Solanum lycopersicum* which suggest that C-26-hydroxyl is the position of oxidation and transamination based on metabolite accumulation using S. *lycopersicum* RNAi lines of genes involved in α-tomatine biosynthesis.

22-Hydroxy-26-aminocholesterol 22-oxidase

[0140] To identify the fourth enzyme in the pathway, a series of quintuple infections of insect cells were carried out that all contained cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase, 22-hydroxycholesterol-26-al transaminase, and *E. californica* CPR, but varied the fourth enzyme. Candidates for the fourth enzyme were the remaining top-scoring candidate cDNAs resulting from interrogation of the *V. californicum* transcriptome dataset (minus the three enzymes already identified). Contig VC13284 also annotated as a steroid C-22 hydroxylase. VC13284 was able to hydroxylate at the 22-position but only slightly above background as detected by LC-MS/MS, but was able to oxidize an existing hydroxyl group at position 22 with much greater efficiency than cholesterol 22-hydroxylase (Figure 3). VC13284 oxidizes the 22-hydroxy position of 22(*R*)-hydroxycholesterol to form 22-keto-cholesterol (Figure 3), 22,26-dihydroxycholesterol to form 22-keto-26-hydroxycholesterol (Figure 3), and 22-hydroxy-26-aminocholesterol to form a short lived intermediate that spontaneously cyclizes to verazine (Figure 4). Four homologs were isolated, each having more than 97% identity and all performing identical reactions. CYP designations for each sequence are CYP90G1v1, CYP90G1v2, CYP90G1v3, and CYP90G2. The structures of the enzymatic products 22-keto-cholesterol and 22-keto-26-hydroxycholesterol were confirmed by NMR spectroscopy. The structure of verazine was confirmed by high-resolution mass

spectrometry (Figure 12). This enzyme was subsequently designated 22-hydroxy-26-aminocholesterol 22-oxidase.

[0141] The recombinant proteins cholesterol 22-hydroxylase, 22-hydroxy-26-aminocholesterol 22-oxidase, and 22-hydroxycholesterol 26-hydroxylase/oxidase (all cytochromes P450) could be detected by SDS-PAGE (Figure 13); however, a band for the gene product of 22-hydroxycholesterol-26-al transaminase (a GABA transaminase) was not observed. To verify the expression data obtained by read mapping, the inventorsperformed semi-quantitative RT-PCR on each functionally identified contig. As seen in Figure 14, the expression patterns found by RNA-seq *vs* semi-quantitative RT-PCR was comparable. The overall pattern is consistent for each gene between both sets of data. These results validate the use of alignment data from the cleaned reads to the assembled contigs to determine relative gene expression.

Biosynthetic pathway to verazine

[0142] The substrate specificities that were determined for the four new enzymes of steroid alkaloid biosynthesis suggested a potential metabolic grid in the metabolism of cholesterol. To determine the likely order of biosynthesis, the following experiments were done. Cholesterol 22-hydroxylase catalyzes the 22-hydroxylation of cholesterol; this is most likely the first step in the biosynthesis of steroid alkaloids in *V. californicum*, confirmed by the inability of 22-hydroxycholesterol 26-hydroxylase/oxidase to hydroxylate cholesterol and very low ability of 22-hydroxy-26-aminocholesterol 22-oxidase to accept a substrate without a C-22 hydroxyl group (Figure 15).

[0143] To establish the pathway order after 22-hydroxylation of cholesterol, a series of enzyme assays were carried out using *S. frugiperda* Sf9 cell extracts containing each cytochrome P450 co-expressed only with *E. californica* CPR (or no co-expression in regards to 22-hydroxycholesterol-26-al transaminase). The order of addition for each enzyme was varied, and products were analyzed by GC-MS or LC-MS/MS. The flow chart for both sets of experiments is presented in Figure 1. For each set of experiments, enzyme assays were extracted (at each arrow in Figure 1) to provide substrate for the next enzyme assay and subsequent enzymes were tested in a systematically varied order.

[0144] Initially, 22-hydroxy-26-aminocholesterol 22-oxidase was incubated with 22(*R*)-hydroxycholesterol to produce 22-keto-cholesterol; the enzymatic product was extracted, and

then tested as substrate with 22-hydroxycholesterol 26-hydroxylase/oxidase. In parallel, 22-hydroxycholesterol 26-hydroxylase/oxidase was incubated with 22(*R*)-hydroxycholesterol to produce 22,26-dihydroxycholesterol; the compound was extracted, and then tested as substrate with 22-hydroxy-26-aminocholesterol 22-oxidase. As seen in Figure 16C; E, 22-keto-26-hydroxycholesterol was only produced at detectable levels by 22-hydroxy-26-aminocholesterol 22-oxidase from 22,26-dihydroxycholesterol. 22-Hydroxycholesterol 26-hydroxylase/oxidase was unable to hydroxylate 22-keto-cholesterol at levels detected by GC-MS. The ability of 22-hydroxy-26-aminocholesterol 22-oxidase to accept 22,26-dihydroxycholesterol as substrate and produce 22-keto-26-hydroxycholesterol, along with the lack of product detection for 22-hydroxycholesterol 26-hydroxylase/oxidase incubated with 22-keto-cholesterol, provided evidence that 22-hydroxycholesterol 26-hydroxylase/oxidase acted directly after cholesterol 22-hydroxylase. This evidence was substantiated with another set of enzyme assays, beginning with cholesterol 22-hydroxylase, showing that 22-hydroxycholesterol 26-hydroxylase/oxidase produced very little product when provided with 22-keto-cholesterol (using the increased sensitivity of LC-MS/MS for detection) as seen in Figure 11B, as compared to the large amount of product produced when 22,26-dihydroxycholesterol is acted upon by 22-hydroxy-26-aminocholesterol 22-oxidase (Figure 11A).

[0145] 22-Hydroxycholesterol-26-al transaminase produced the same product in the presence or absence of 22-hydroxy-26-aminocholesterol 22-oxidase (Figure 4A; C). 22-Hydroxycholesterol-26-al transaminase, therefore, did not require a 22-ketone moiety on the substrate. When cholesterol 22-hydroxylase acted in the presence of 22-hydroxycholesterol 26-hydroxylase/oxidase, several side products were made in addition to 22,26-dihydroxycholesterol (Figure 11A). This included 22-keto-26-hydroxycholesterol, 22-hydroxycholesterol-26-al and two other products. Since an amino group was not added to the 22-ketone moiety of 22-keto-26-hydroxycholesterol, 22-keto-26-hydroxycholesterol most likely does not participate in the steroid alkaloid pathway. The short lived and highly reactive 22-hydroxycholesterol-26-al must be the substrate of the 22-hydroxycholesterol-26-al transaminase. Once the amino group is transferred to the C-26 aldehyde, 22-hydroxy-26-aminocholesterol 22-oxidase oxidizes the C-22-hydroxyl moiety to a ketone, and cyclization to verazine spontaneously occurs (Figure 4C).

[0146] Evidence of the short-lived intermediate 22-hydroxycholesterol-26-al was obtained with a dimedone aldehyde trapping experiment (Figure 17). The dimedone derivative could

not be identified, however, a reduction in enzymatic product was observed in the presence of dimedone. The amino group is added prior to oxidation of the C-22 hydroxyl group, therefore the amino group must be transferred to the C-26 aldehyde. The structure of the predicted cyclic imine verazine was supported by borohydride reduction of the double bond (Figure 18) and exact mass analysis as demonstrated by high resolution MS (Figure 12).

[0147] The biosynthetic pathway proposed herein (Figure 5) is consistent with an hypothesized pathway presented in earlier studies of steroidal alkaloids in the genus *Veratrum*. In further support of the proposed pathway, selected biosynthetic intermediates were detected in *V. californicum* extracts by LC-MS/MS (Figure 6). The accumulation of these intermediates follows the same pattern as cyclopamine (Figure 2). Verazine has also been detected in steroid alkaloid producing *Veratrum* species and was previously hypothesized an intermediate in steroidal alkaloid biosynthesis.

Site of steroid alkaloid biosynthesis in *V. californicum*

[0148] A comparison was made between biosynthetic gene expression profiles and cyclopamine accumulation in *V. californicum* (Figure 7). A pattern emerged that indicates that biosynthetic genes are most highly expressed in root, spring rhizome and bulb tissue, while steroid alkaloid accumulation is highest in spring and fall rhizome. Moreover, the higher level of cyclopamine in fall rhizome compared to spring rhizome indicates an accumulation of the steroid alkaloid during rhizome growth in summer.

[0149] Figure 6 shows a comparison of the relative quantity of each detectable intermediate to the transcript level of the corresponding biosynthetic enzyme. Each gene expression value and metabolite accumulation value is expressed as a percent of the total for comparison. Interestingly, in all cases, the percent of gene expression and metabolite accumulation are similar in root and spring rhizome, but fall rhizome has significantly more metabolite relative to transcript level; the opposite is true for bulb, comparable with the accumulation pattern of cyclopamine. The biosynthetic intermediates were below limits of detection in bulb, so transport of these metabolites is plausible. Accumulation of these metabolites in fall rhizome and low gene expression suggests the rhizome may be used in metabolite storage for the plant. *Veratrum californicum* rarely seeds and new growth is mainly established by rhizome, generating evolutionary pressure for its protection.

Evolution of steroid alkaloid biosynthesis

[0150] Recently, a biosynthetic pathway was proposed for steroid glycoalkaloids in *S. lycopersicum*. The pathway shares many similar reactions as the proposed cyclopamine pathway in *V. californicum* (Figure 5), but some key differences emerge. In *S. lycopersicum*, initial transformations of cholesterol include C-22 hydroxylation followed by C-26 hydroxylation and closure of the E-ring. Oxidation at C-26, then transamination at that position occurs next. In conjunction with our results, previous work on steroid alkaloid formation in *Veratrum* does not support E-ring closure prior to aldehyde formation and transamination. Verazine production requires formation of the F-ring following transamination; prior to E-ring closure. If the pathway was identical to that proposed in *S. lycopersicum*, the E-ring closure prior to amination would not allow for the formation of verazine.

[0151] The contrasting pathways may be explained by the phylogenetic relationship of these enzymes. Figure 8 shows a phylogenetic analysis of select cytochrome P450 enzymes including several involved in steroid metabolism and Figure 9 shows the phylogenetic relationship of selected plant GABA transaminases. Cholesterol 22-hydroxylase in *V. californicum* and GAME7, the proposed cholesterol 22-hydroxylase in *S. lycopersicum*, do not share a recent common ancestor and appear evolutionarily distinct. Each shows only 20% identity at the amino acid level; the same for 26-hydroxylase in *S. lycopersicum* (GAME8) and *V. californicum* 22-hydroxycholesterol 26-hydroxylase/oxidase. GAME4, the *S. lycopersicum* enzyme that performs oxidation at position 26 does not cluster near 22-hydroxy-26-aminocholesterol 22-oxidase or 22-hydroxycholesterol 26-hydroxylase/oxidase of *V. californicum.* These relationships do not appear to be due to the evolutionary distinction between monocot and eudicots, as *V. californicum* cholesterol 22-hydroxylase and 22-hydroxy-26-aminocholesterol 22-oxidase share common ancestor cytochrome P450 enzymes from both classes of plants. Both of these enzymes cluster closer to the CYP90B1s from *Arabidopsis thaliana* and *S. lycopersicum*. The *Arabidopsis* CYP90B1 was shown to hydroxylate cholesterol, as well as other steroids in brassinosteroid metabolism. The relationship of the *V. californicum* enzymes and the CYP90B1s may be indicative of *Veratrum* alkaloid biosynthesis evolution deriving from the brassinosteroid pathway.

[0152] The phylogenetic relationship of GABA transaminases shows evidence of a potential polyploidy event that led to the duplication and subsequent neo-functionalization of the GABA transaminase genes. The *V. californicum* 22-hydroxycholesterol-26-al transaminase that incorporates nitrogen into 22-hydroxycholesterol-26-al does not cluster closely with the

S. lycopersicum GABA transaminase isozyme 2 involved in steroid alkaloid biosynthesis as seen in Figure 9, despite 64% identity. Due to the potential function and sequence homology, the inventors decided to test whether *S. lycopersicum* GABA transaminase isozyme 2 can incorporate nitrogen into 22-hydroxycholesterol-26-al. As seen in Figure 4, it was demonstrated that *S. lycopersicum* GABA transaminase isozyme 2 was able to transaminate 22-hydroxycholesterol-26-al to 22-hydroxy-26-aminocholesterol with subsequent cyclization to verazine. The *S. lycopersicum* GABA transaminase isozyme 2 was used as query to BLAST the *V. californicum* transcriptome, and interestingly, the best hit was another transaminase, contig VC674. VC674, designated GABA transaminase 2, annotated as a GABA transaminase with 68% identity to *S. lycopersicum* GABA transaminase isozyme 2 and 69% identity to *V. californicum* 22-hydroxycholesterol-26-al transaminase at the amino acid level. Despite the homology to *V. californicum* 22-hydroxycholesterol-26-al transaminase and *S. lycopersicum* GABA transaminase isozyme 2, GABA transaminase 2 was unable to catalyze the reaction (Figure 19). The *V. californicum* 22-hydroxycholesterol-26-al transaminase shows closer homology to other monocot GABA transaminases and to *Amborella*, which predates the monocot/eudicot division. These phylogenetic trees support a unique and independent evolution of the pathway to steroid alkaloids in *Veratrum* compared to tomato.

[0153] The *S. lycopersicum* genes recently identified in steroid alkaloid biosynthesis were found to cluster on chromosomes 7 and 12. Homologs in potato were also found to cluster. Although the genome sequence of *V. californicum* is not yet available, it would facilitate identification of the remainder of the pathway should these biosynthetic genes also cluster.

[0154] Besides engineering the cyclopamine and/or verazine-derived metabolite pathway(s) in higher plants and algae in order to obtain cyclopamine and/or verazine-derived metabolites economically and in high yield, the present disclosure also encompasses cyclopamine and/or verazine-derived metabolite production in plant cell cultures, cell-free extracts, production in organisms such as transgenic fungi, yeasts, bacteria such as *E. coli* and *B. subtilis*, and the use of immobilized enzymes, etc. In certain embodiments, the methods and compounds of the present disclosure may be used to regulate proliferation of cells and/or cell death in vitro and/or in vivo such as in the treatment of malignant disorders of the head, neck, nasal cavity, paranasal sinuses, nasopharynx, oral cavity, oropharynx, larynx, hypopharynx, salivary glands, paragangliomas, pancreas, stomach, skin, esophagus, liver and biliary tree, bone,

intestine, colon, rectum, ovaries, prostate, lung, breast, lymphatic system, blood, bone marrow central nervous system, or brain.

[0155] Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the disclosure specifically described herein. Such equivalents are intended to be encompassed within the scope of the following claims.

What Is Claimed Is:

1. A transgenic plant or a transgenic organism that produces a biochemical compound, wherein the biochemical compound is selected from the group consisting of cyclopamine and verazine-derived metabolite.

2. The transgenic plant or the transgenic organism of claim 1, comprising within its genome, and expressing, a heterologous nucleotide sequence, wherein the heterologous nucleotide sequence encodes for an enzyme, wherein the enzyme is selected from the group consisting of a cytochrome P450 enzyme and a γ-aminobutyrate transaminase.

3. The transgenic plant or the transgenic organism of claim 2, wherein said nucleotide sequence is selected from among SEQ ID NOs:1-26.

4. The transgenic plant or the transgenic organism of claim 3, selected from the group consisting of a species of *Brachypodium*, a species of *Setaria*, a species of *Populus*, tobacco, corn, rice, soybean, cassava, canola (rapeseed), wheat, peanut, palm, coconut, safflower, sesame, cottonseed, sunflower, flax, olive, safflower, sugarcane, castor bean, switchgrass, *Miscanthus*, *Camelina* and *Jatropha*.

5. The transgenic plant or the transgenic organism of claim 4, wherein said heterologous nucleotide sequence is codon-optimized for expression in said transgenic plant.

6. The transgenic plant or the transgenic organism of claim 5, wherein said heterologous nucleotide sequence is expressed in a tissue or organ selected from among an inflorescence, a flower, a sepal, a petal, a pistil, a stigma, a style, an ovary, an ovule, an embryo, a receptacle, a seed, a fruit, a stamen, a filament, an anther, a male or female gametophyte, a pollen grain, a meristem, a terminal bud, an axillary bud, a leaf, a stem, a root, a tuberous root, a rhizome, a tuber, a stolon, a corm, a bulb, an offset, a cell of said plant in culture, a tissue of said plant in culture, an organ of said plant in culture, and a callus.

7. A method of making a transgenic plant or a transgenic organism, comprising the steps of:

(i) inserting into the genome of a plant cell or an organism cell a heterologous nucleotide sequence comprising, operably linked for expression:

(a) a promoter sequence;

(b) at least one heterologous nucleotide sequence coding for an enzymes, wherein the enzyme is selected from the group consisting of a cytochrome P450 enzyme and a γ-aminobutyrate transaminase;

(ii) obtaining a transformed plant cell or a transformed organism cell; and

(iii) regenerating from said transformed plant cell or said transformed organism cell a genetically transformed plant or a genetically transformed organism, wherein said genetically transformed plant or said genetically transformed organism produces a biochemical compound, wherein the biochemical compound is selected from the group consisting of cyclopamine and a verazine-derived metabolite.

8. A pharmaceutical composition consisting of a biochemical compound, wherein said biochemical compound is selected from the group consisting of cyclopamine and a verazine-derived metabolite, wherein said biochemical compound is a recovered from a transgenic plant or a transgenic organism.

9. The pharmaceutical composition of claim 8, wherein the transgenic plant or transgenic is made by a method comprising the steps of:

(i) inserting into the genome of a plant cell or an organism cell a heterologous nucleotide sequence comprising, operably linked for expression:

(a) a promoter sequence;

(b) at least one heterologous nucleotide sequence coding for an enzymes, wherein the enzyme is selected from the group consisting of a cytochrome P450 enzyme and a γ-aminobutyrate transaminase;

(ii) obtaining a transformed plant cell or a transformed organism cell; and

(iii) regenerating from said transformed plant cell or said transformed organism cell a genetically transformed plant or a genetically transformed organism, wherein said genetically transformed plant or said genetically transformed organism produces a biochemical compound, wherein the biochemical compound is selected from the group consisting of cyclopamine and a verazine-derived metabolite.

10. The pharmaceutical composition of claim 9, further comprising a pharmaceutically acceptable carrier, dilient, or excipient.

11. The pharmaceutical composition of claim 10, wherein the pharmaceutical composition is for use in a method for the treatment of cancer.

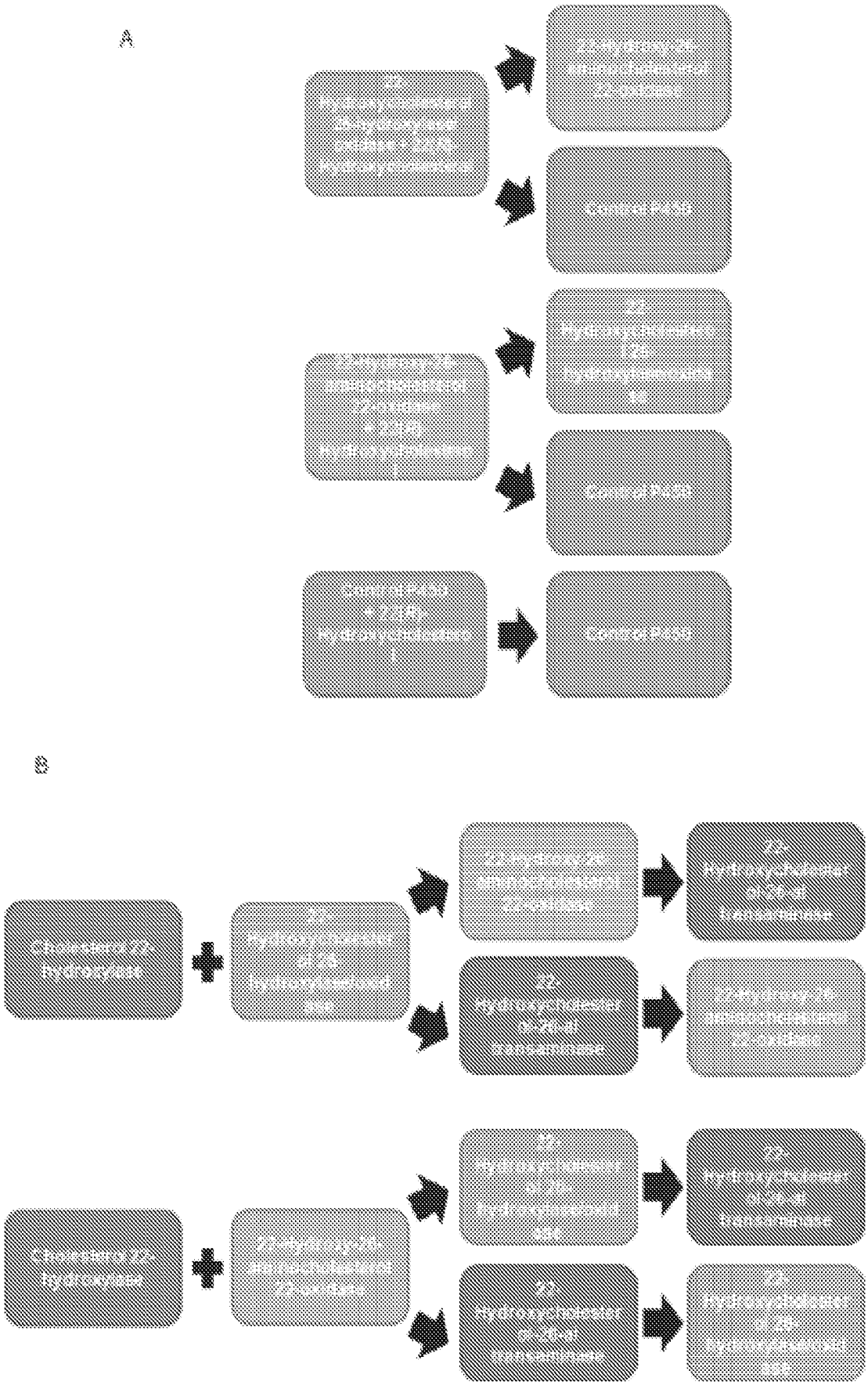

A
Control Peptide
Control Peptide
Control Peptide
Control Peptide
B
Control Peptide
Control Peptide
Control Peptide
Control Peptide

Figure 1

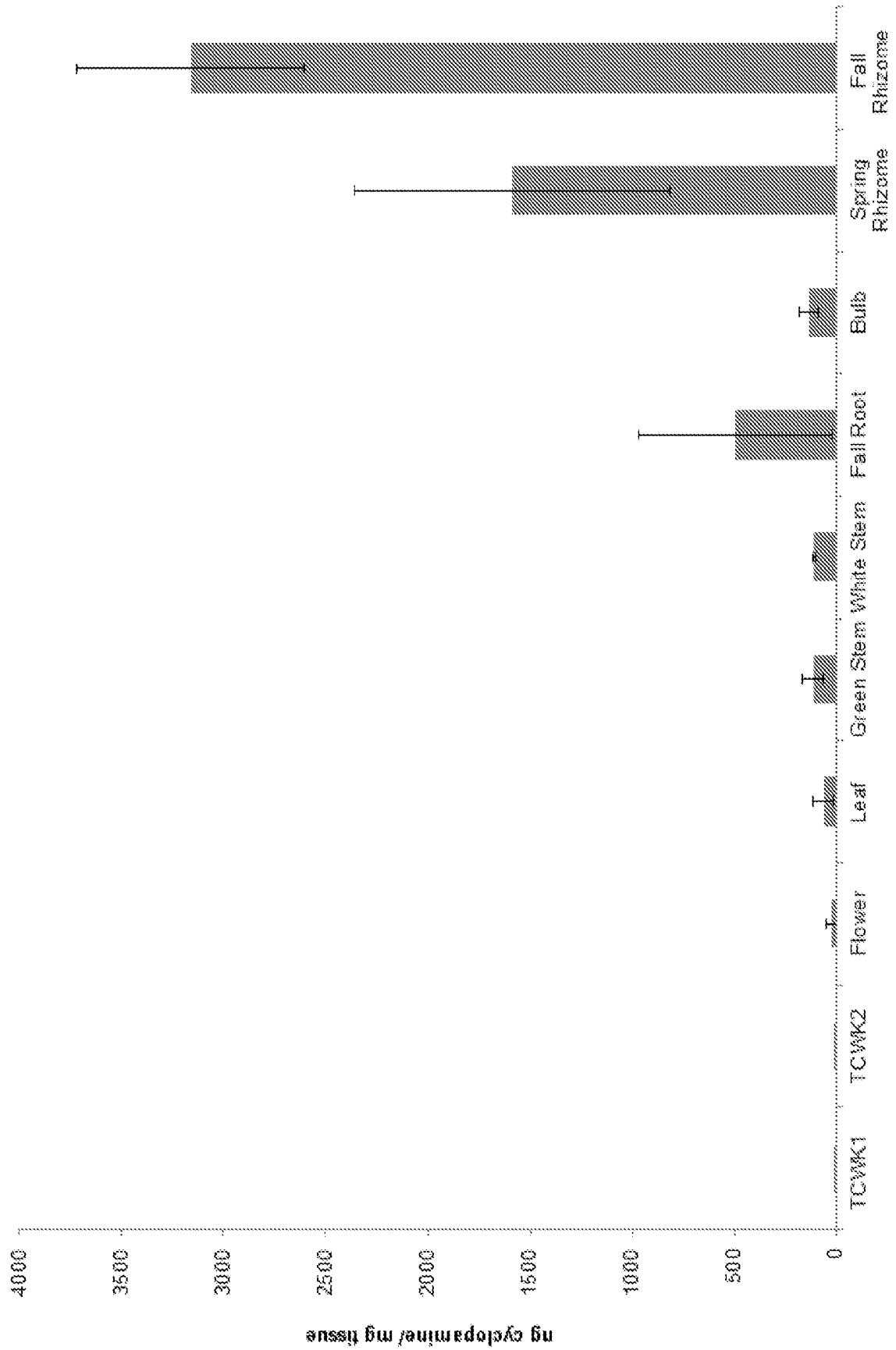

ng cyclopamine/ mg tissue
4000
3500
3000
2500
2000
1500
1000
500
0
TCWK1
TCWK2
Flower
Leaf
Green Stem
White Stem
Fall Root
Bulb
Spring Rhizome
Fall Rhizome

Figure 2

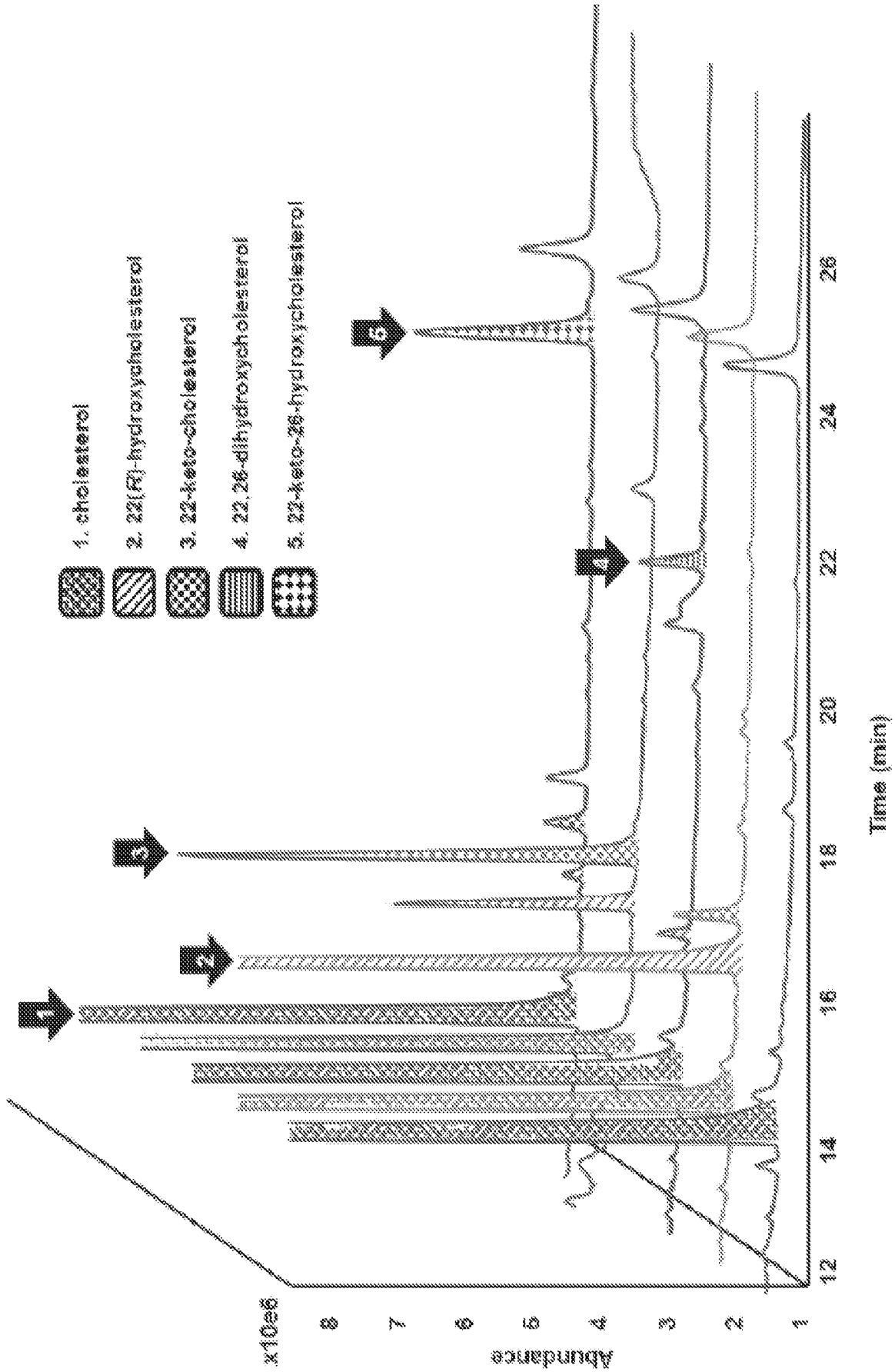

1. cholesterol
2. 22(R)-hydroxycholesterol
3. 22-keto-cholesterol
4. 22,26-dihydroxycholesterol
5. 22-keto-26-hydroxycholesterol
Abundance
x10e6
Time (min)

Figure 3

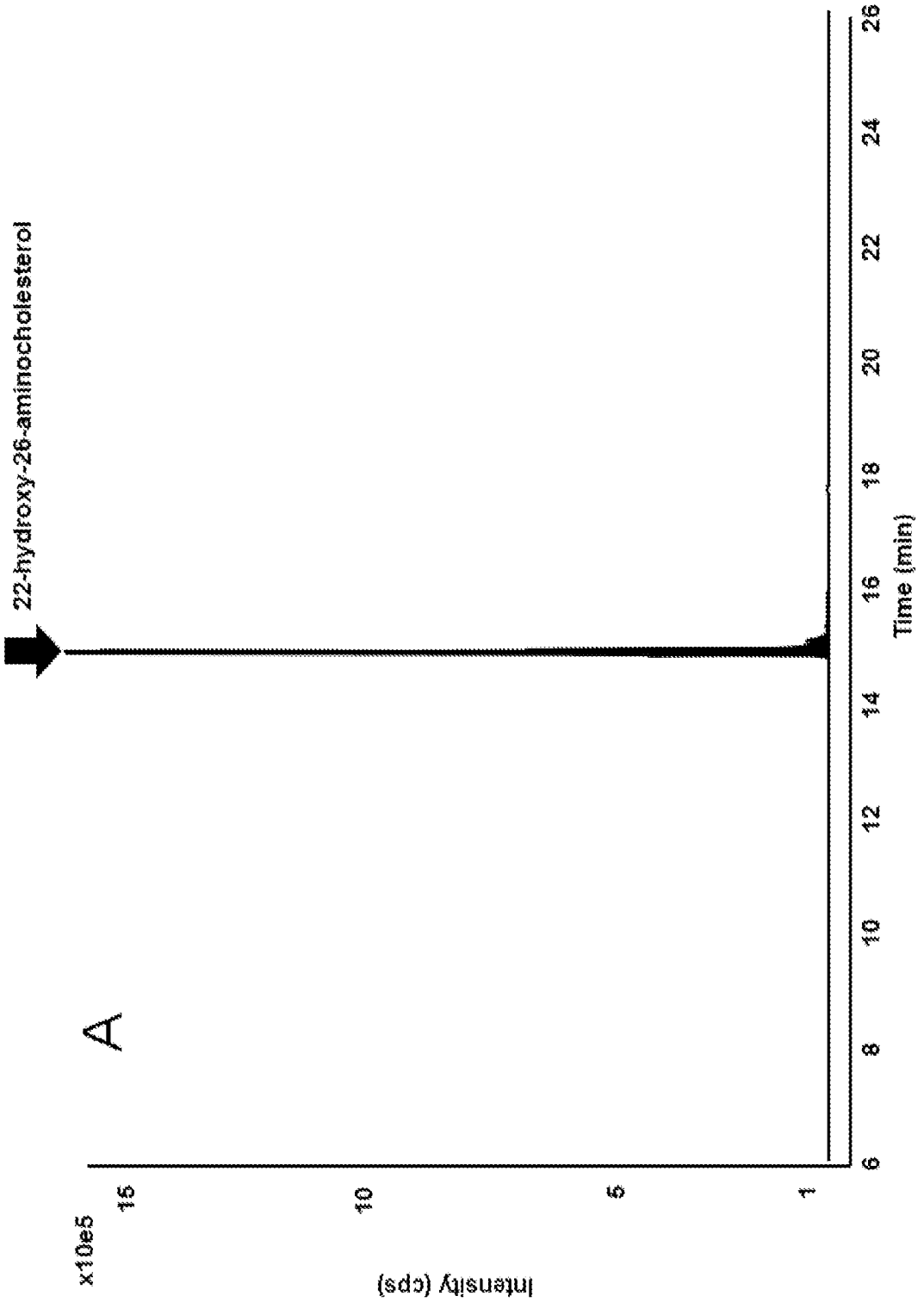

22-hydroxy-26-aminocholesterol
A
x10e5
15
10
5
1
Intensity (cps)
6
8
10
12
14
16
18
20
22
24
26
Time (min)

Figure 4A

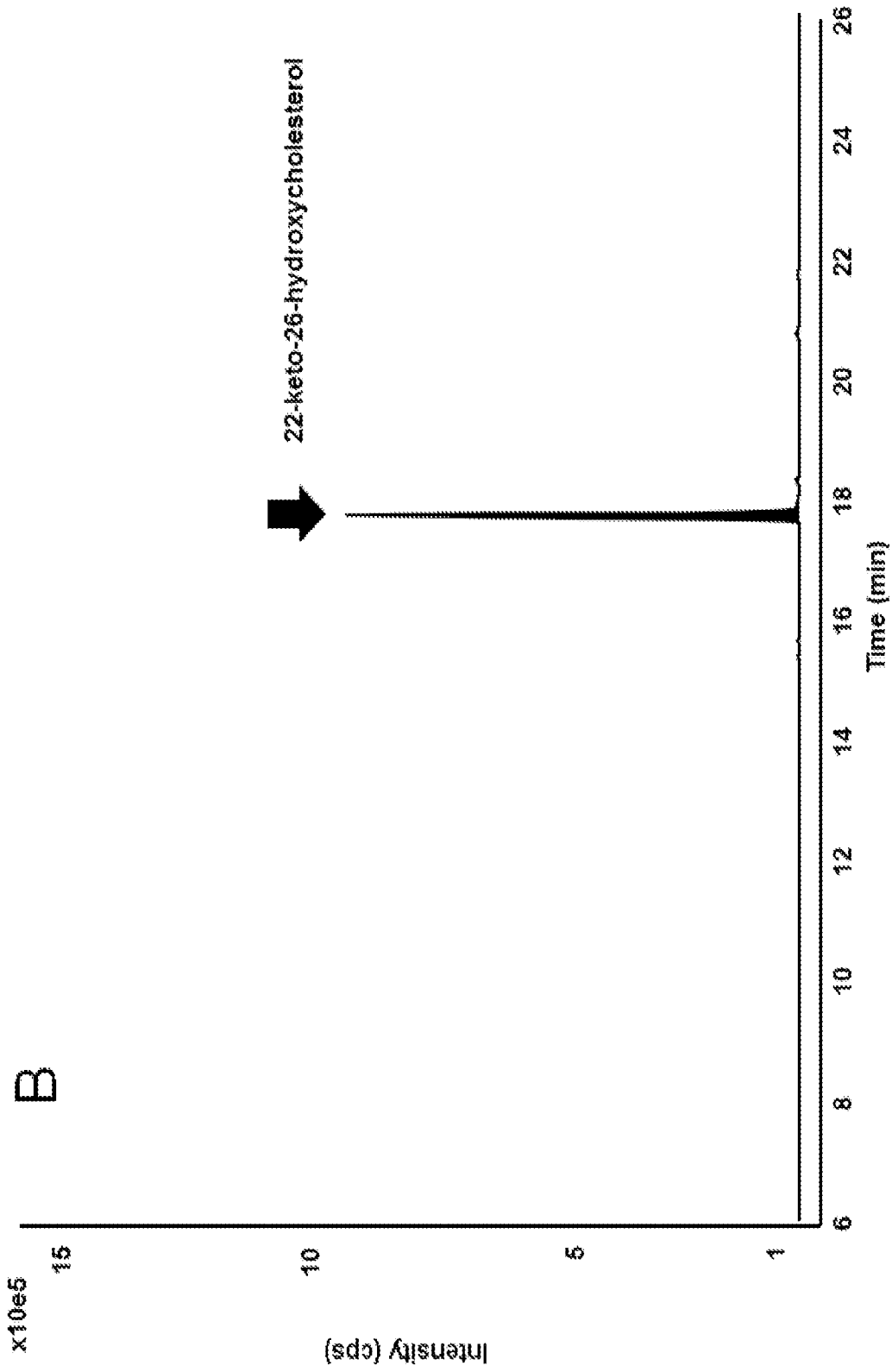

B
22-keto-26-hydroxycholesterol
x10e5
15
10
5
1
Intensity (cps)
6
8
10
12
14
16
18
20
22
24
26
Time (min)

Figure 4B

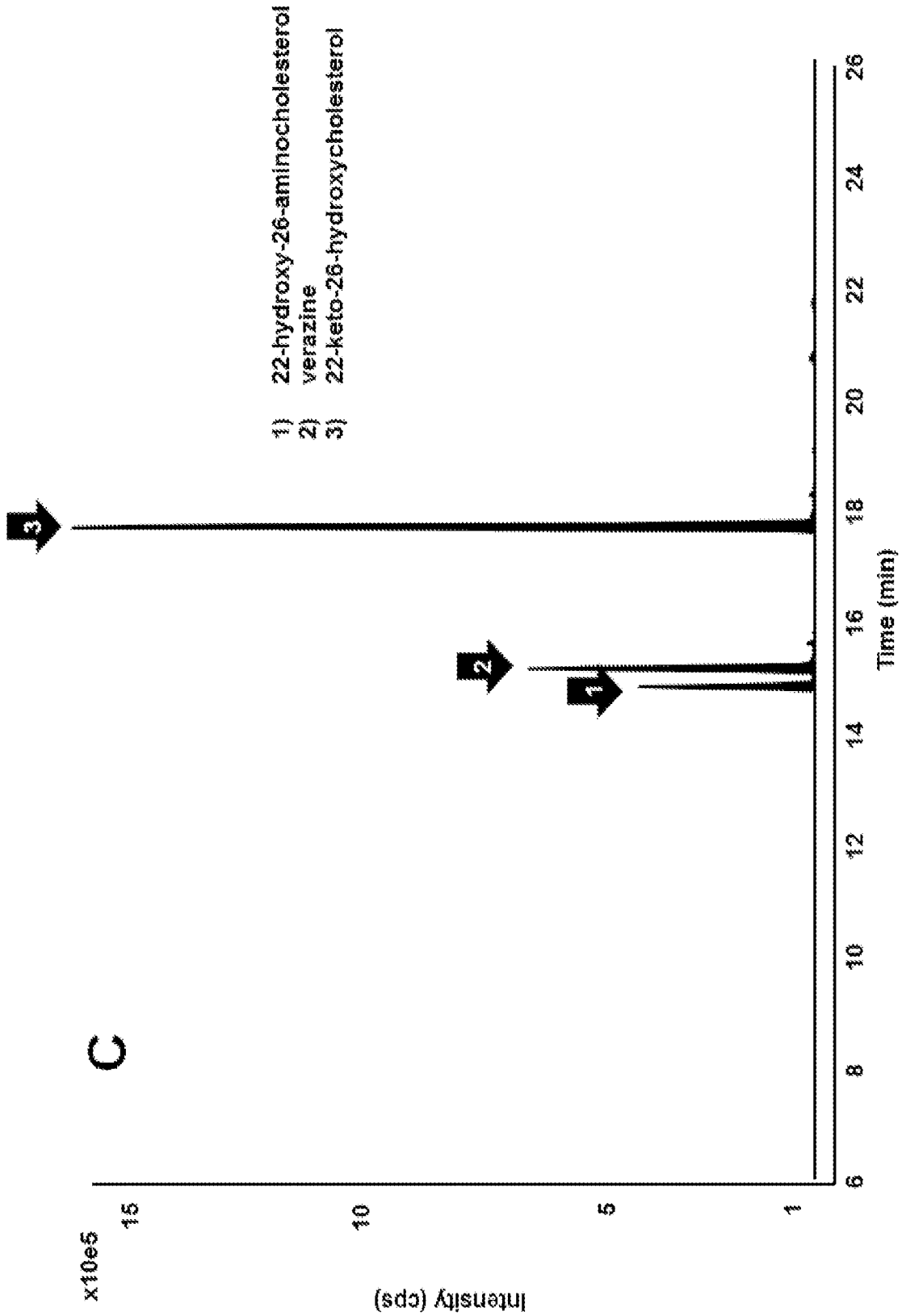

C
1) 22-hydroxy-26-aminocholesterol
2) verazine
3) 22-keto-26-hydroxycholesterol
x10e5
15
10
5
1
Intensity (cps)
6
8
10
12
14
16
18
20
22
24
26
Time (min)
1
2
3

Figure 4C

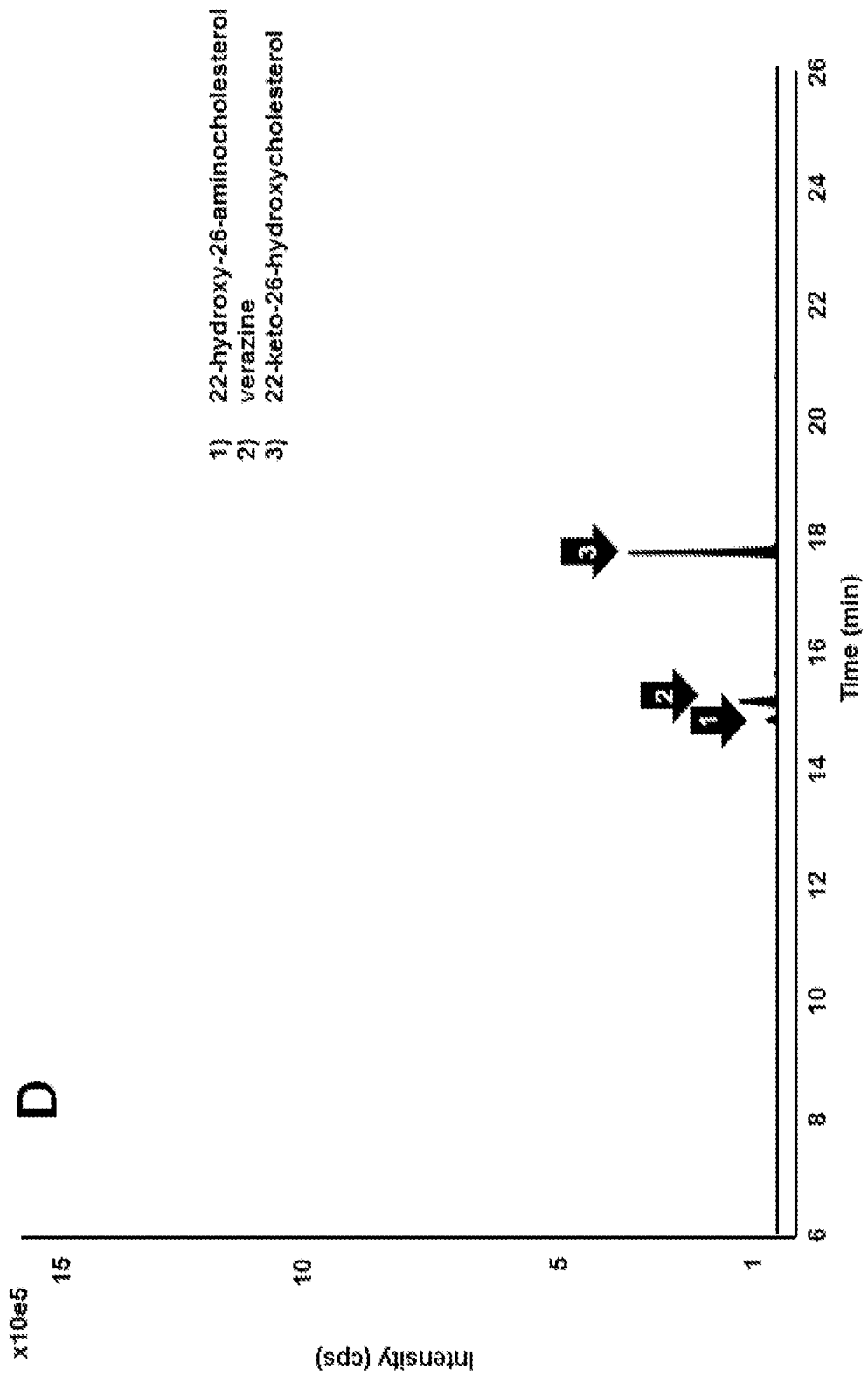

D
x10e5
15
10
5
1
Intensity (cps)
6
8
10
12
14
16
18
20
22
24
26
Time (min)
1
2
3
1) 22-hydroxy-26-aminocholesterol
2) verazine
3) 22-keto-26-hydroxycholesterol

Figure 4D

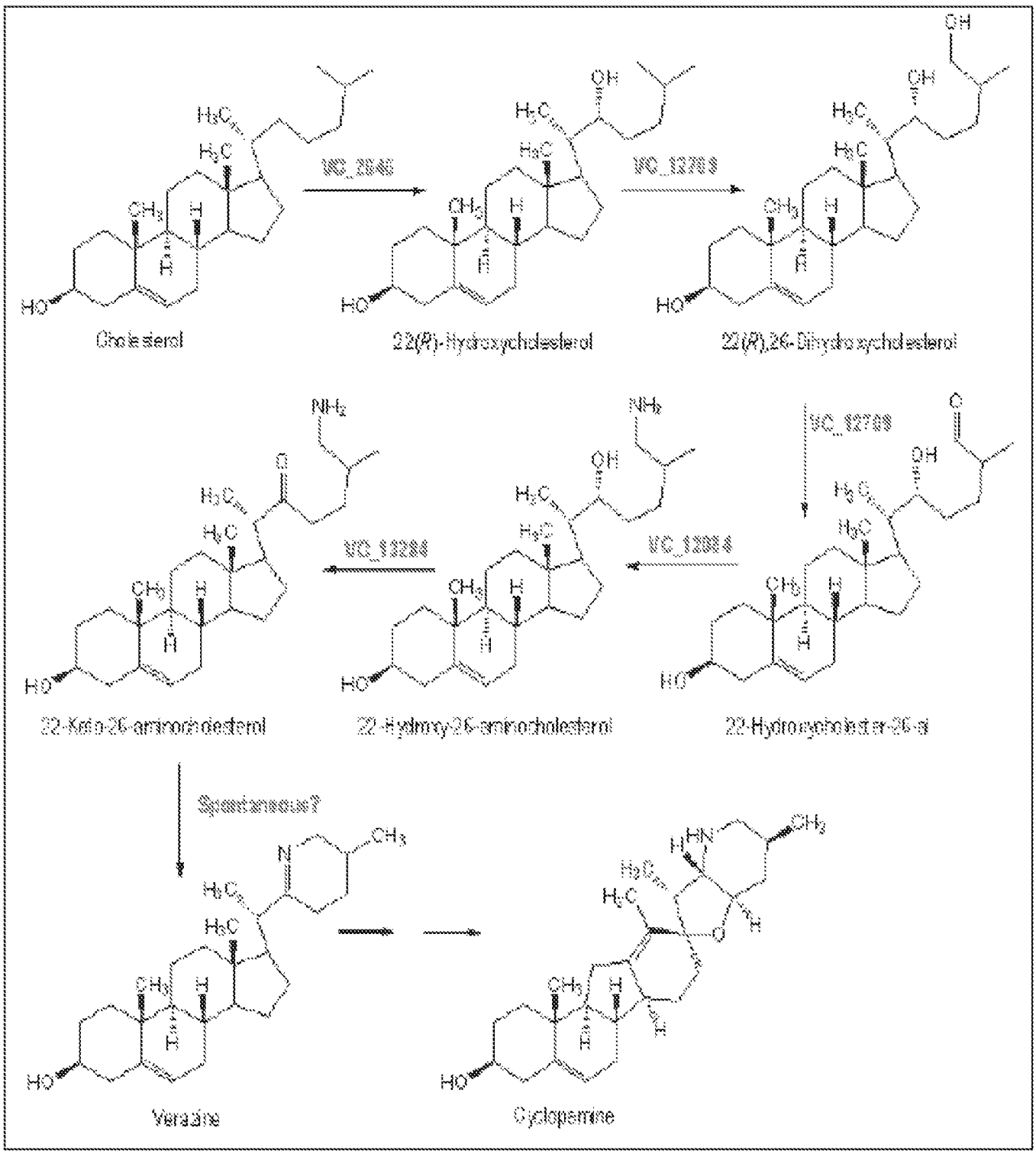

Cholesterol
22(R)-Hydroxycholesterol
22(R),26-Dihydroxycholesterol
22-Keto-26-aminocholesterol
22-Hydroxy-26-aminocholesterol
Spontaneous?
Veratrine
Cyclopamine

Figure 5

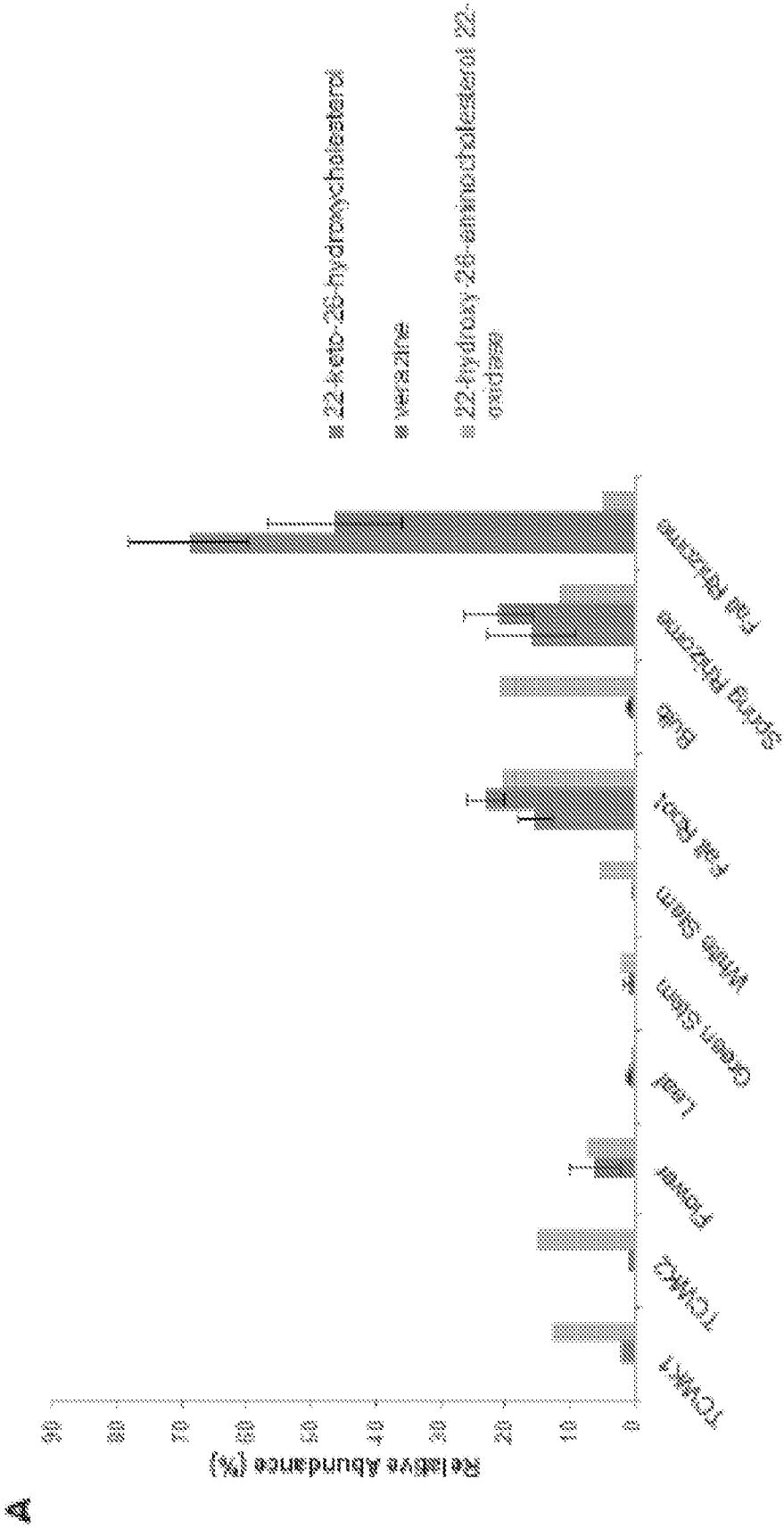

22-keto-26-hydroxycholesterol
veratrine
22-hydroxy-26-aminocholesterol (22-epimer)
Relative abundance (%)
0
10
20
30
40
50
60
70
80
90
TCHRC1
TCHRC2
Prostate
Liver
Green Colon
White Colon
Fat Tissue
Brain
Spinal Marrow
Full Organism

Figure 6A

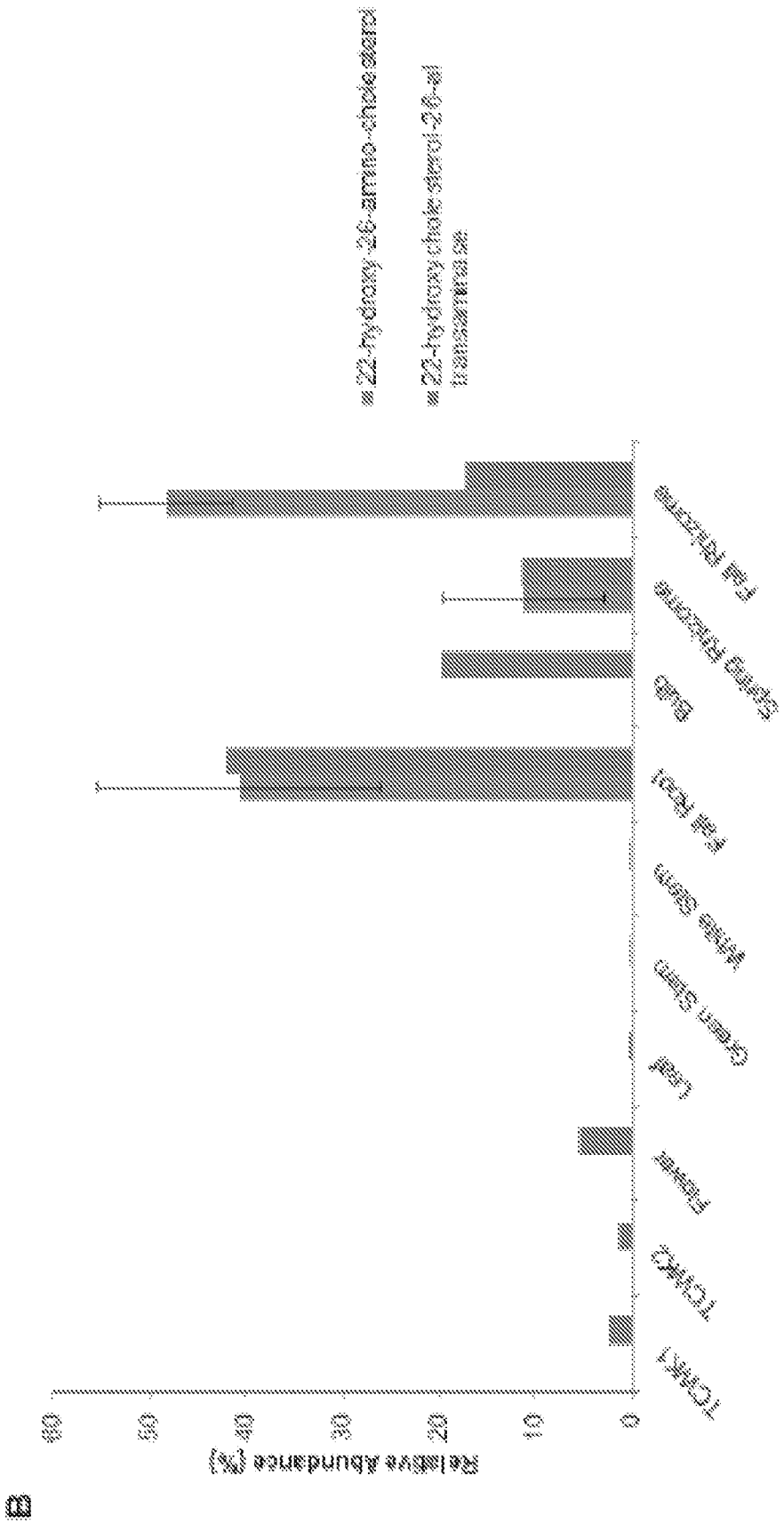

B
Relative abundance (%)
0
10
20
30
40
50
60
TCHK1
TCHK2
Flower
Leaf
Green Stem
White Stem
Fully Open
Opening Epicarp
Bud
Fully Epicarp
22-hydroxy-26-amino-cholestanol
22-hydroxy cholestanol-2,6-el
transaminase

Figure 6B

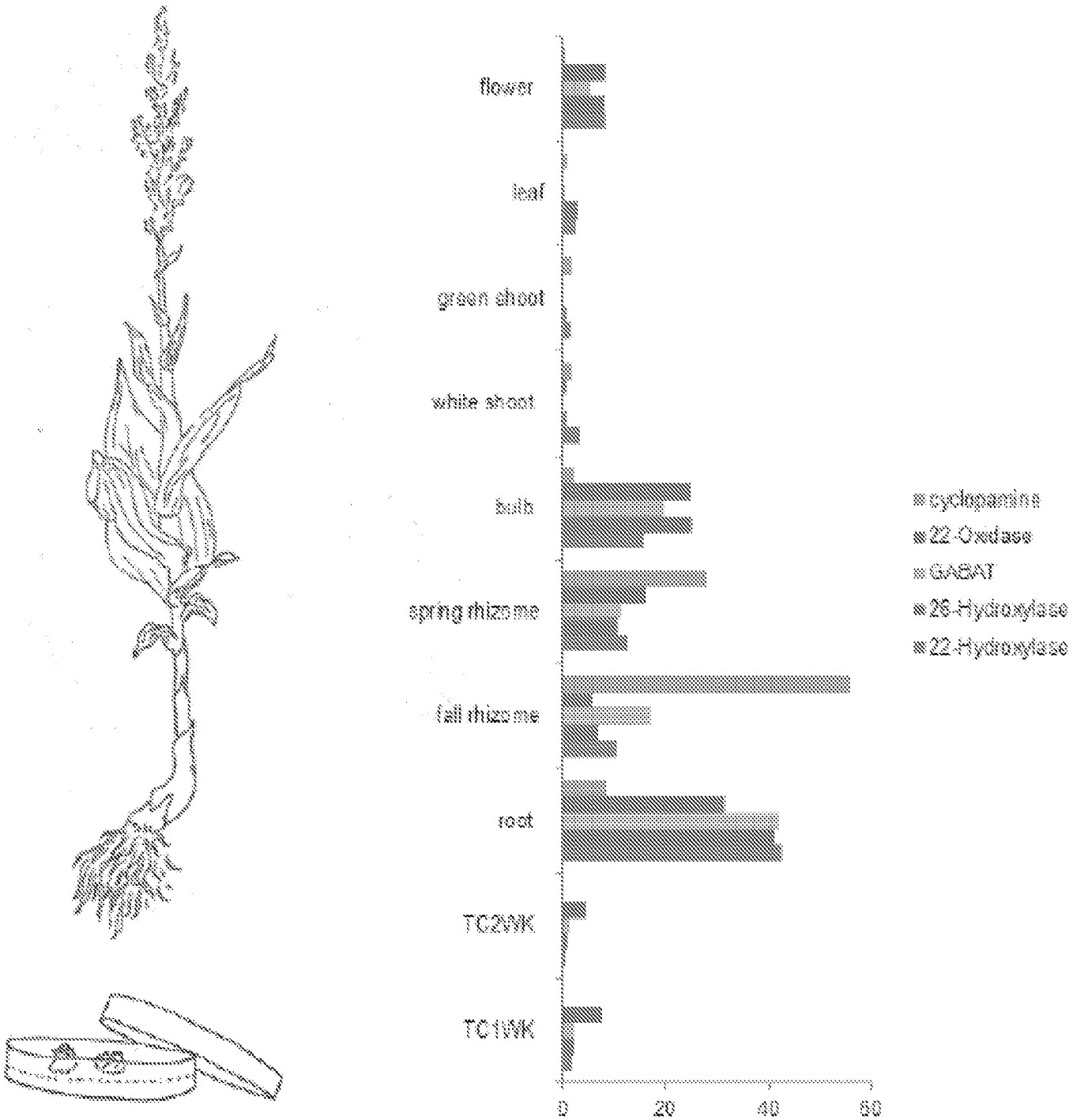

flower
leaf
green shoot
white shoot
bulb
spring rhizome
fall rhizome
root
TC2WK
TC1WK
0
20
40
60
cyclopamine
22-Oxidase
GABAT
26-Hydroxylase
22-Hydroxylase

Figure 7

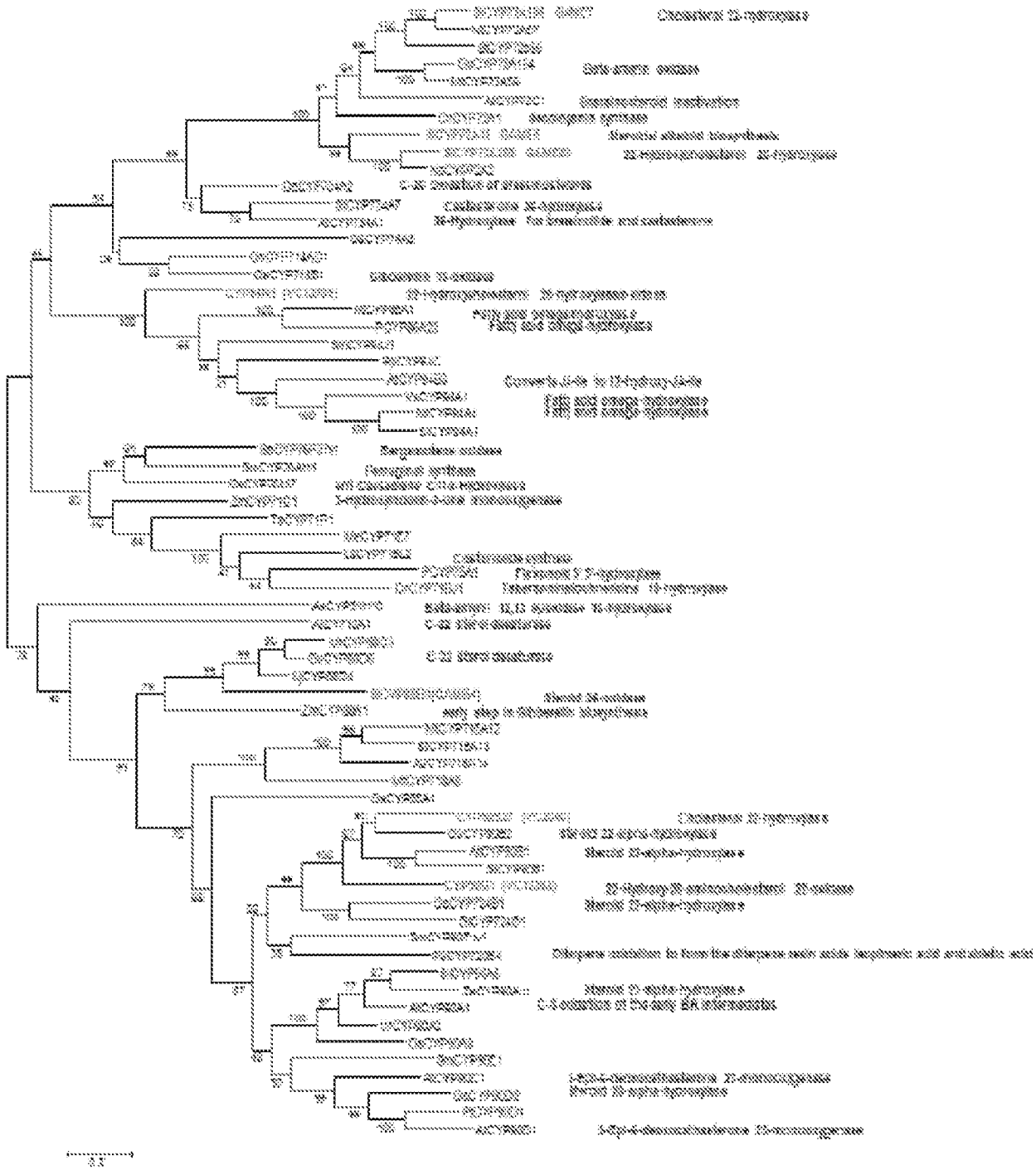


Figure 8

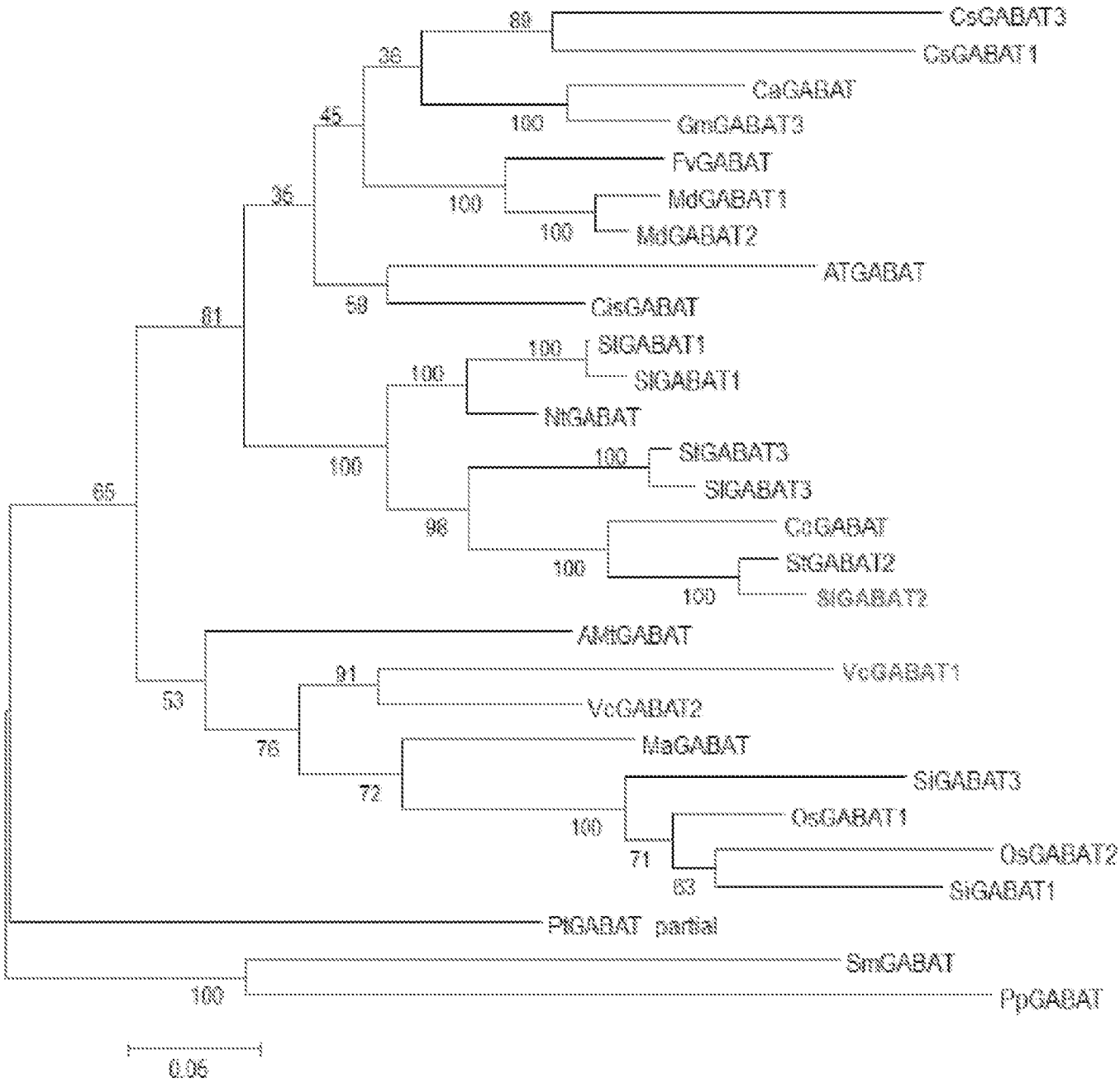

CsGABAT3
CsGABAT1
CaGABAT
GmGABAT3
FvGABAT
MdGABAT1
MdGABAT2
ATGABAT
CisGABAT
SlGABAT1
SlGABAT1
NtGABAT
SlGABAT3
SlGABAT3
CaGABAT
SlGABAT2
SlGABAT2
AMtGABAT
VcGABAT1
VcGABAT2
MaGABAT
SlGABAT3
OsGABAT1
OsGABAT2
SlGABAT1
PtGABAT partial
SmGABAT
PpGABAT
0.06

Figure 9

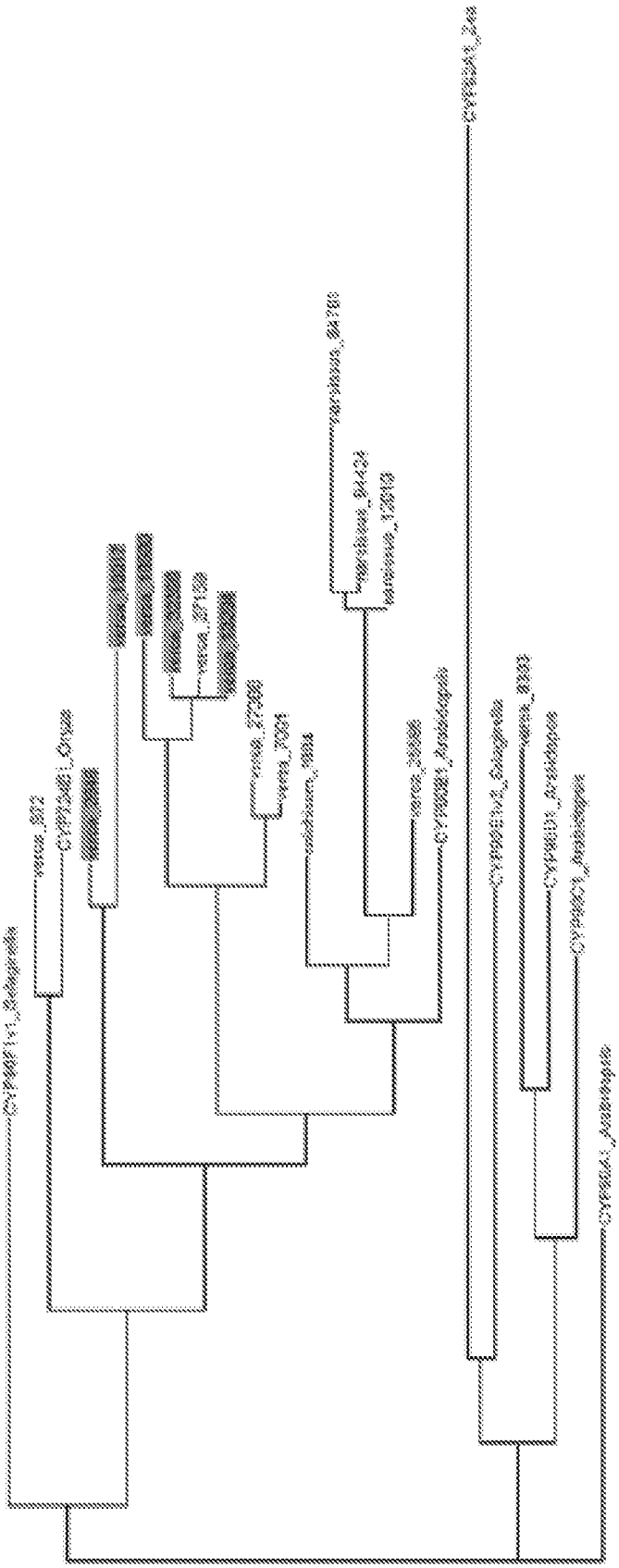


Figure 10

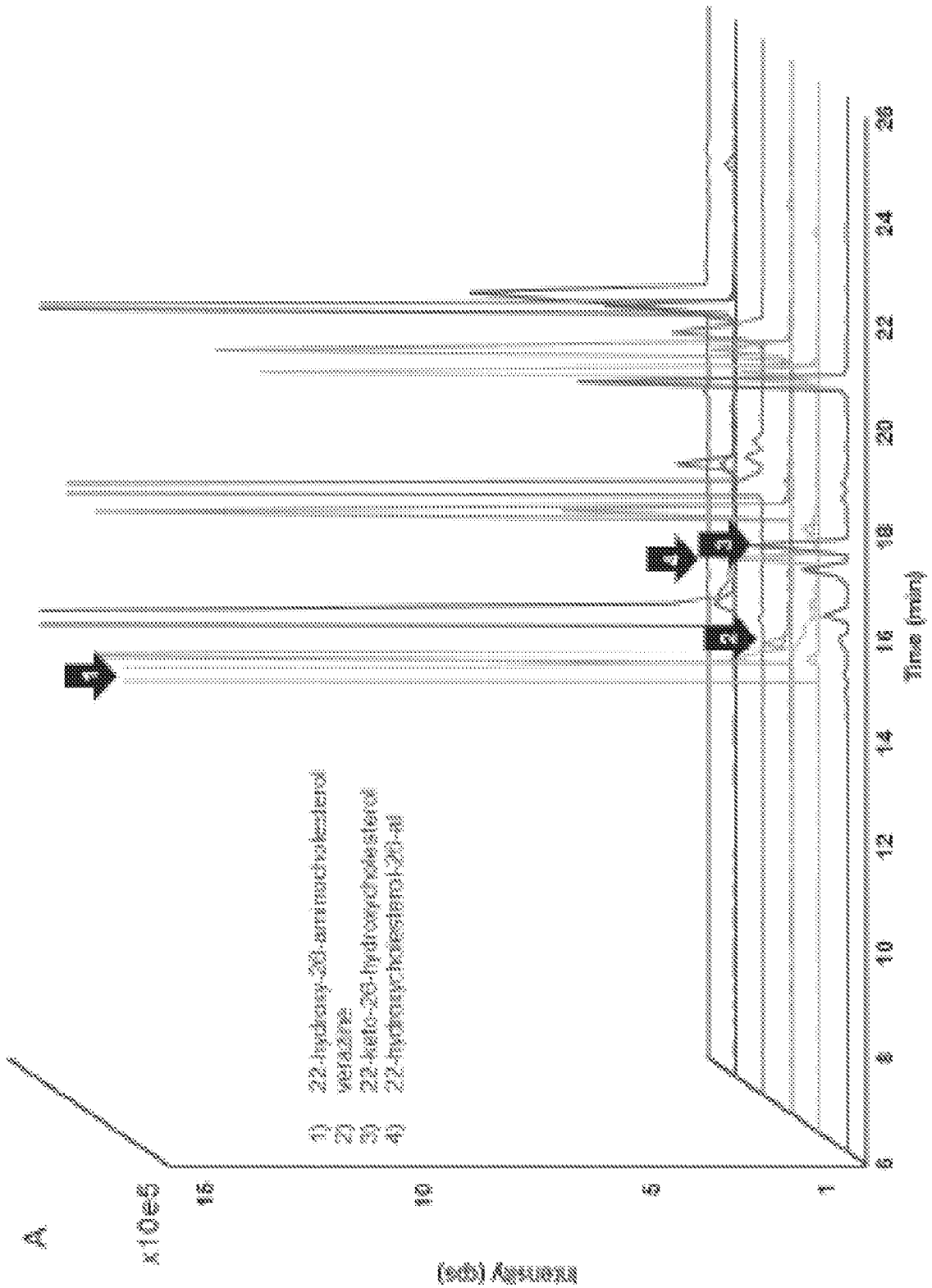

A
x10e5
15
10
5
1
(+ESI) Aguacate
1) 22-hydroxy-20-aminocholesterol
2) veratrine
3) 22-keto-26-hydroxycholesterol
4) 22-hydroxycholesterol-20-al
6
8
10
12
14
16
18
20
22
24
26
Time (min)

Figure 11A

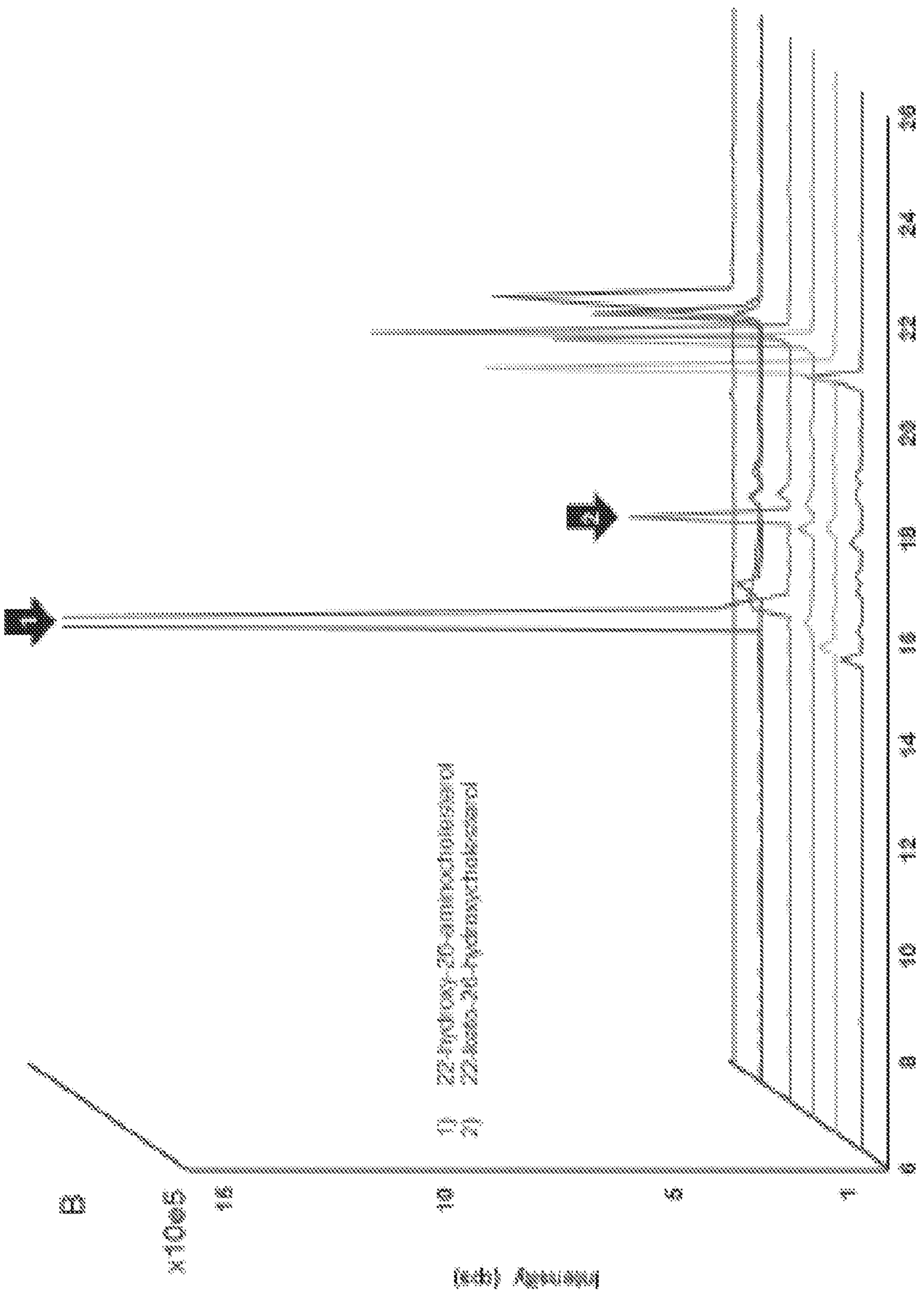


Figure 11B

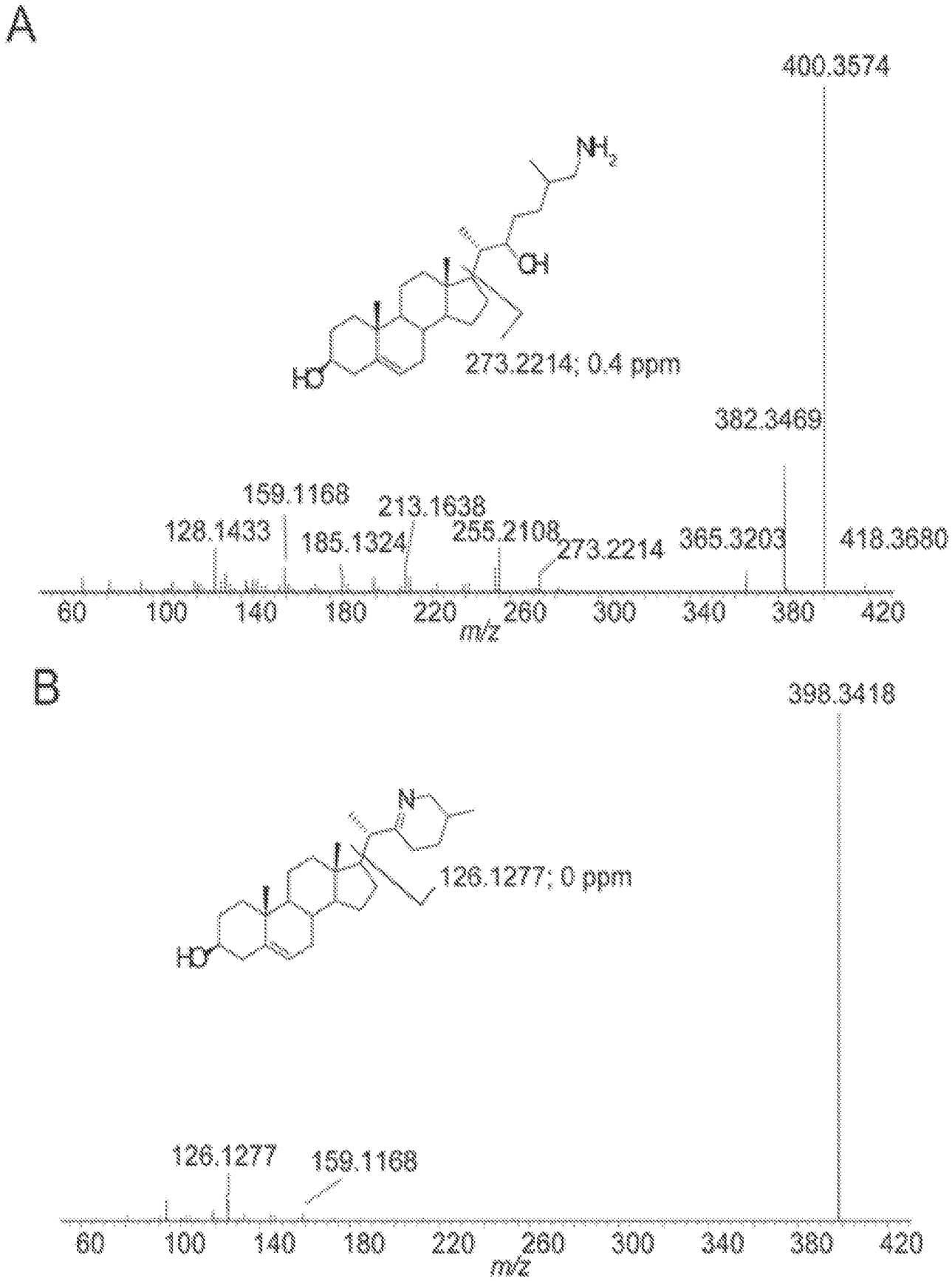

A
400.3574
NH2
OH
HO
273.2214; 0.4 ppm
382.3469
159.1168
213.1638
128.1433
185.1324
255.2108
273.2214
365.3203
418.3680
60
100
140
180
220
260
300
340
380
420
m/z
B
398.3418
N
126.1277; 0 ppm
HO
126.1277
159.1168
60
100
140
180
220
260
300
340
380
420
m/z

Figure 12

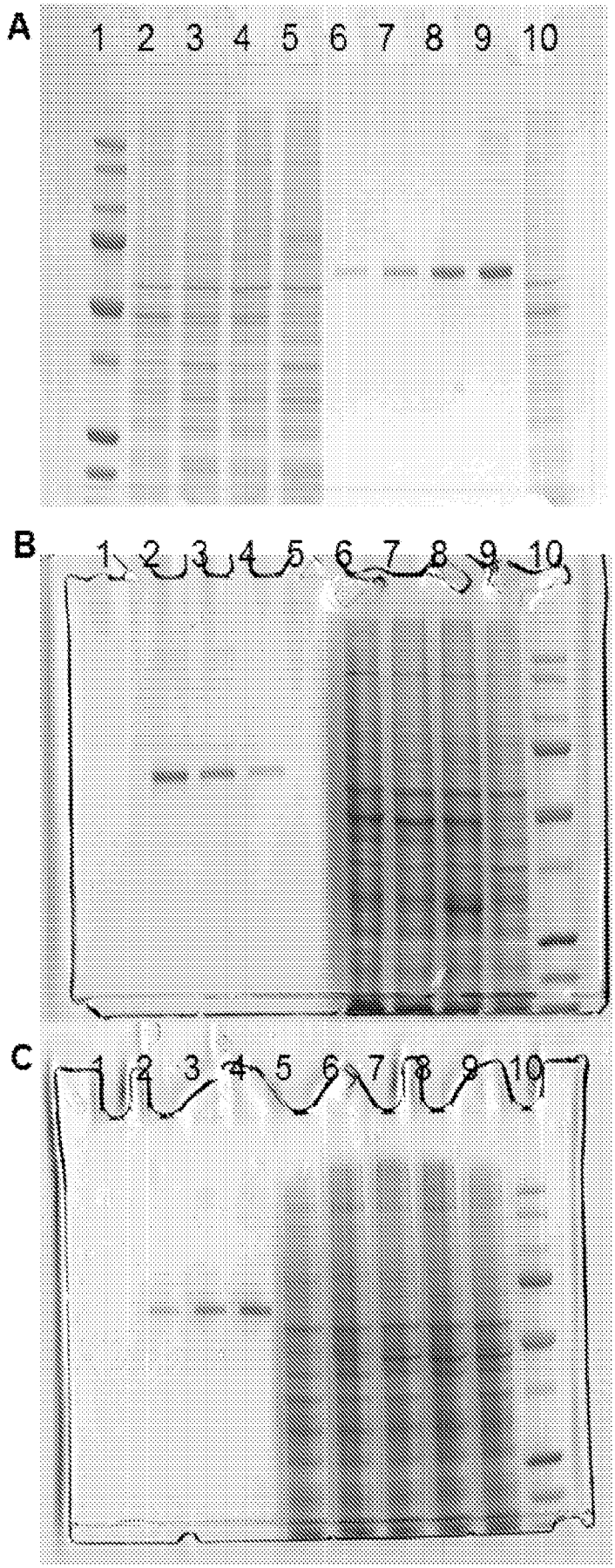


Figure 13

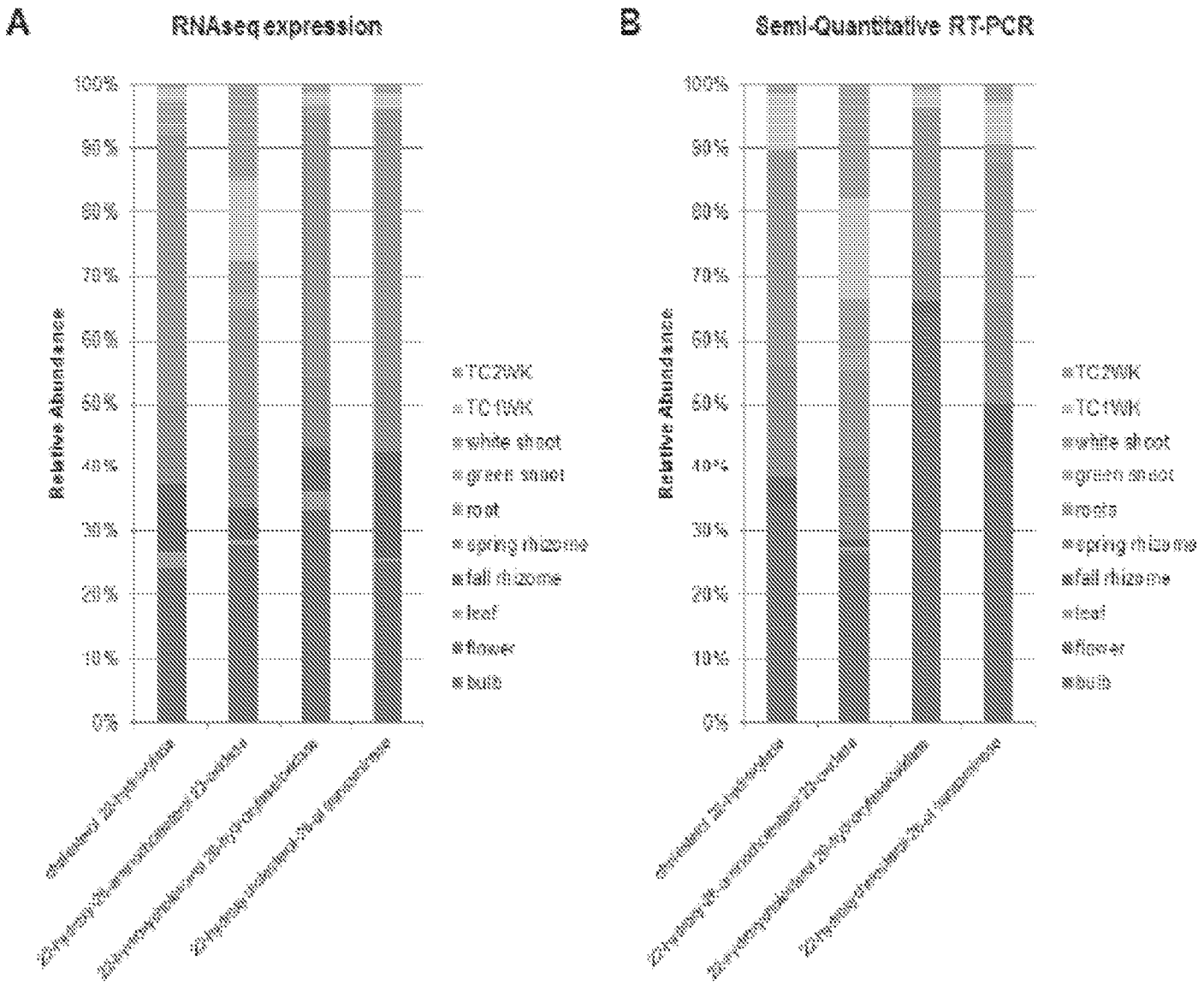

A
RNAseq expression
B
Semi-Quantitative RT-PCR
Relative Abundance
100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
TC29NK
TC19NK
white shoot
green shoot
root
spring rhizome
fall rhizome
leaf
flower
bulb

Figure 14

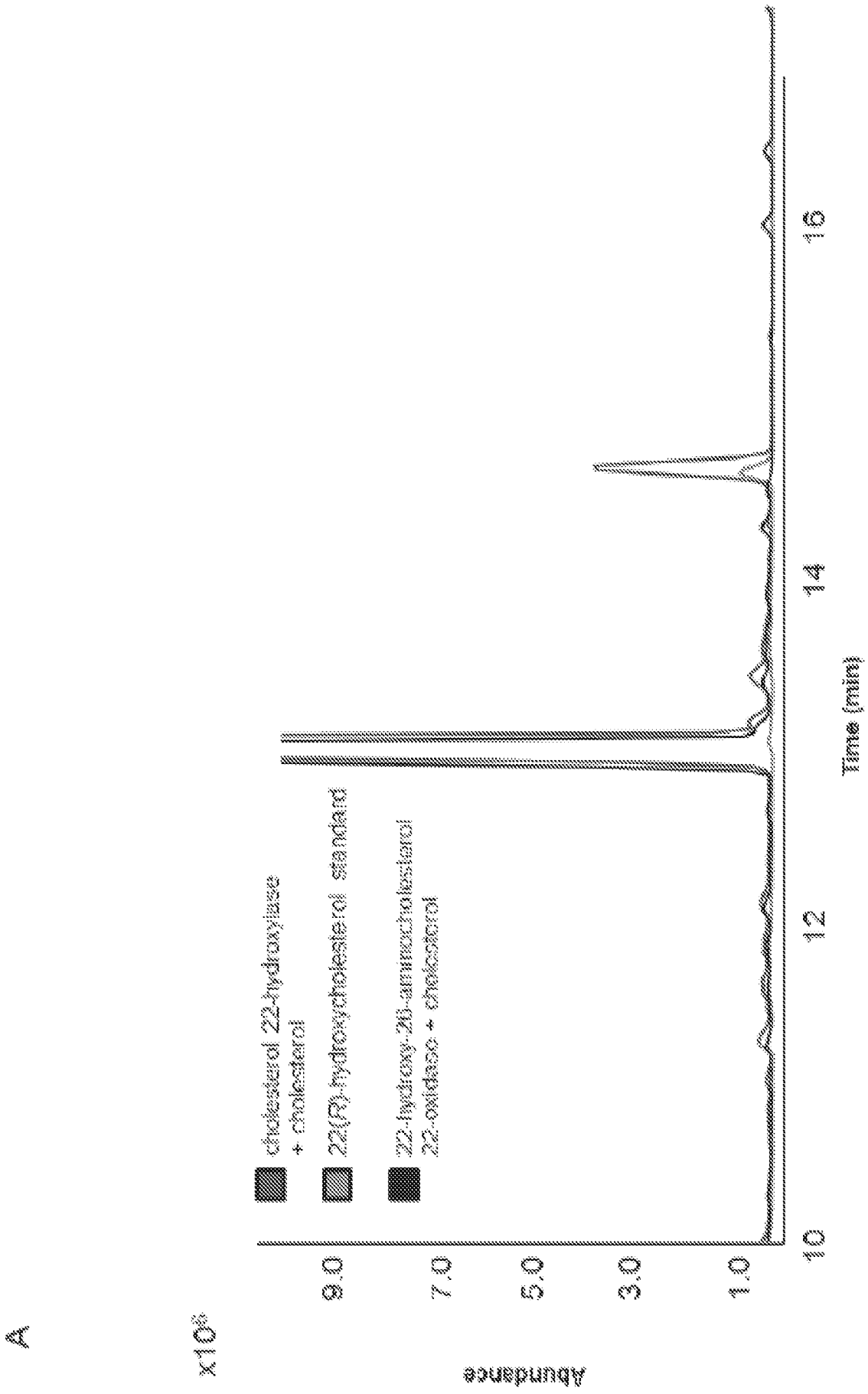

A
x10^6
Abundance
9.0
7.0
5.0
3.0
1.0
10
12
14
16
Time (min)
cholesterol 22-hydroxylase + cholesterol
22(R)-hydroxycholesterol standard
22-hydroxy-26-aminocholesterol 22-oxidase + cholesterol

Figure 15A

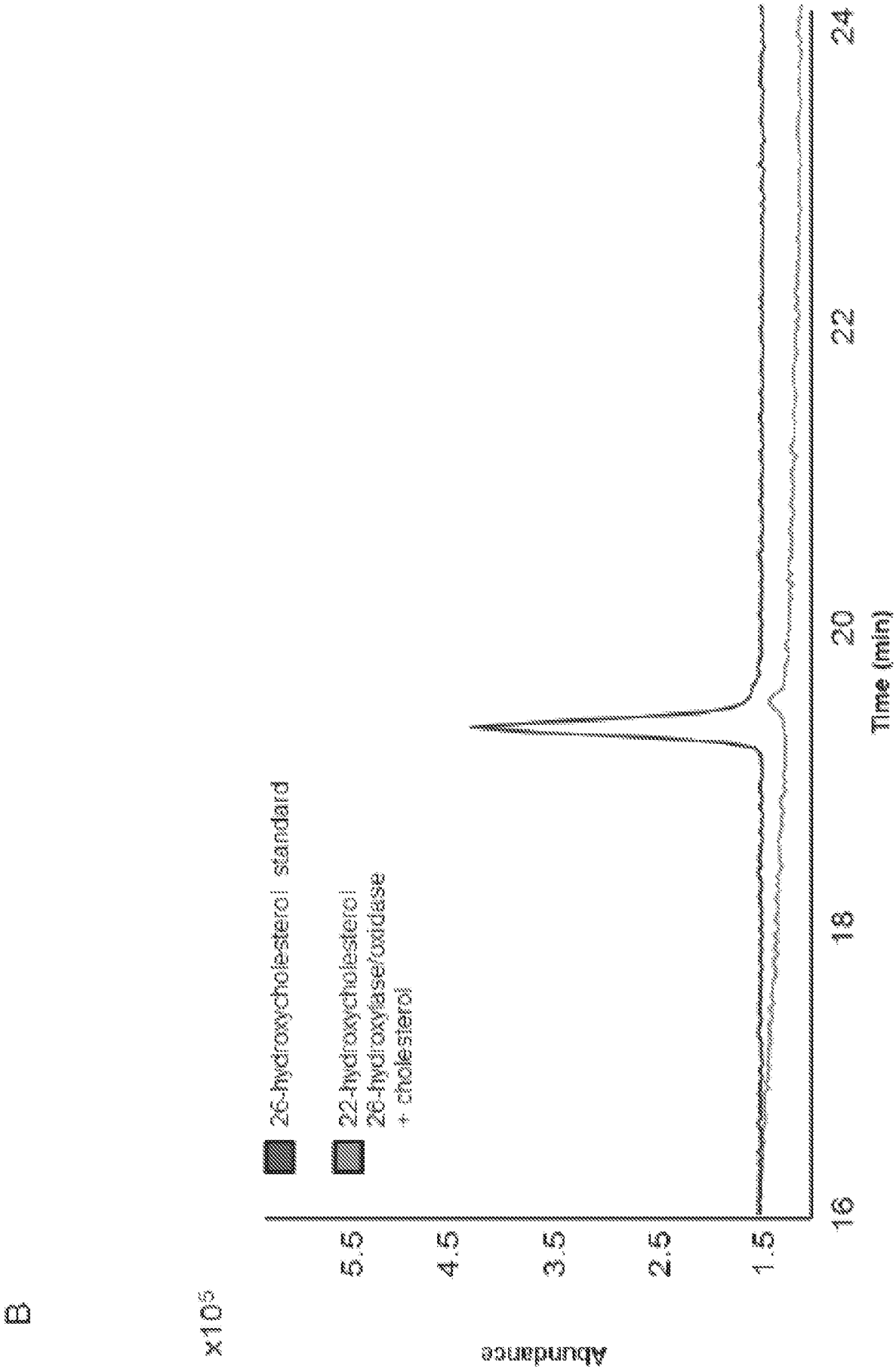

B
x10^5
Abundance
5.5
4.5
3.5
2.5
1.5
16
18
20
22
24
Time (min)
26-hydroxycholesterol standard
22-hydroxycholesterol
26-hydroxylase/oxidase
+ cholesterol

Figure 15B

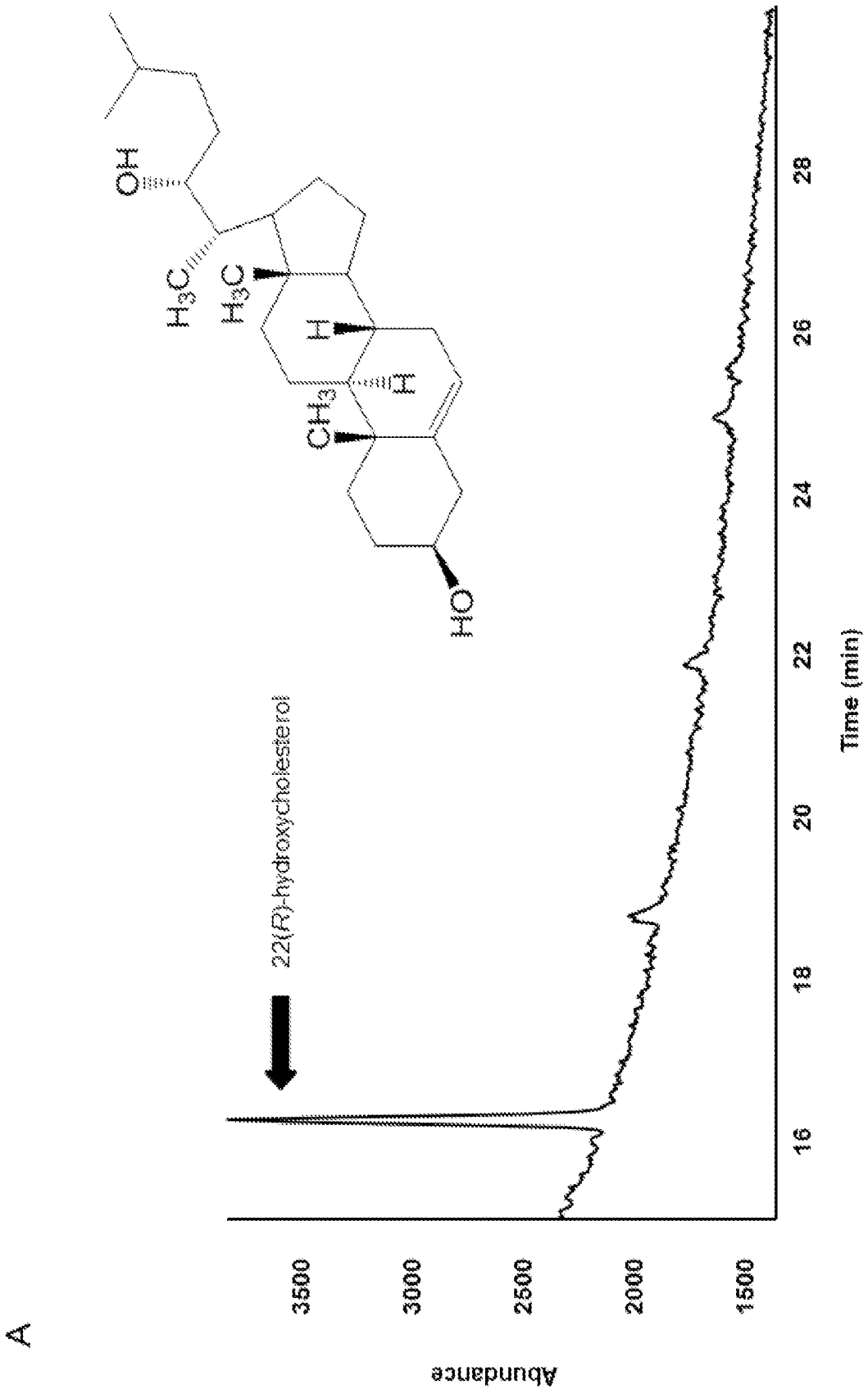

A
Abundance
3500
3000
2500
2000
1500
16
18
20
22
24
26
28
Time (min)
22(R)-hydroxycholesterol
OH
H3C
H3C
H
CH3
H
HO

Figure 16A

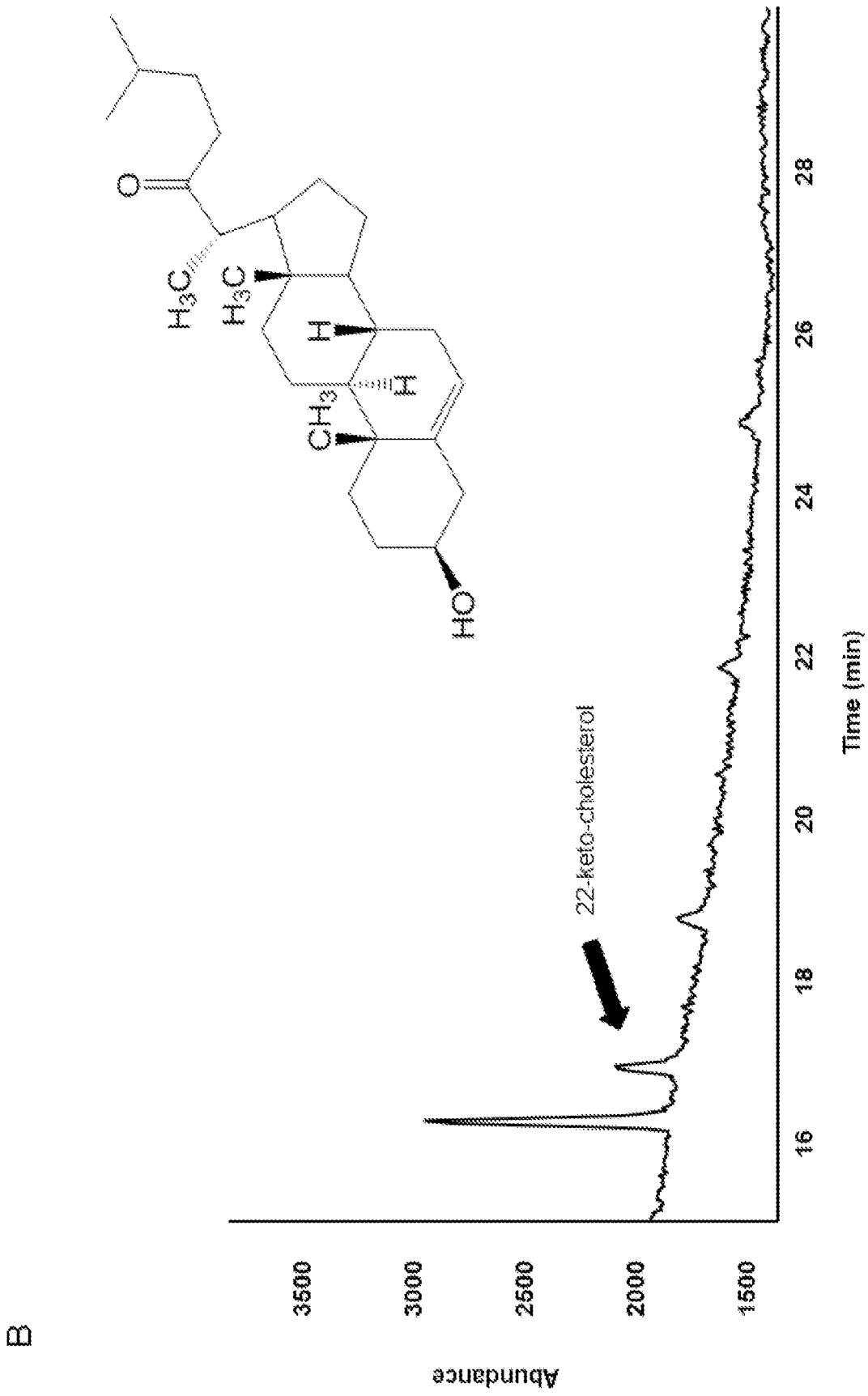

B
Abundance
3500
3000
2500
2000
1500
16
18
20
22
24
26
28
Time (min)
22-keto-cholesterol
HO
H
H
CH3
H3C
H3C
O

Figure 16B

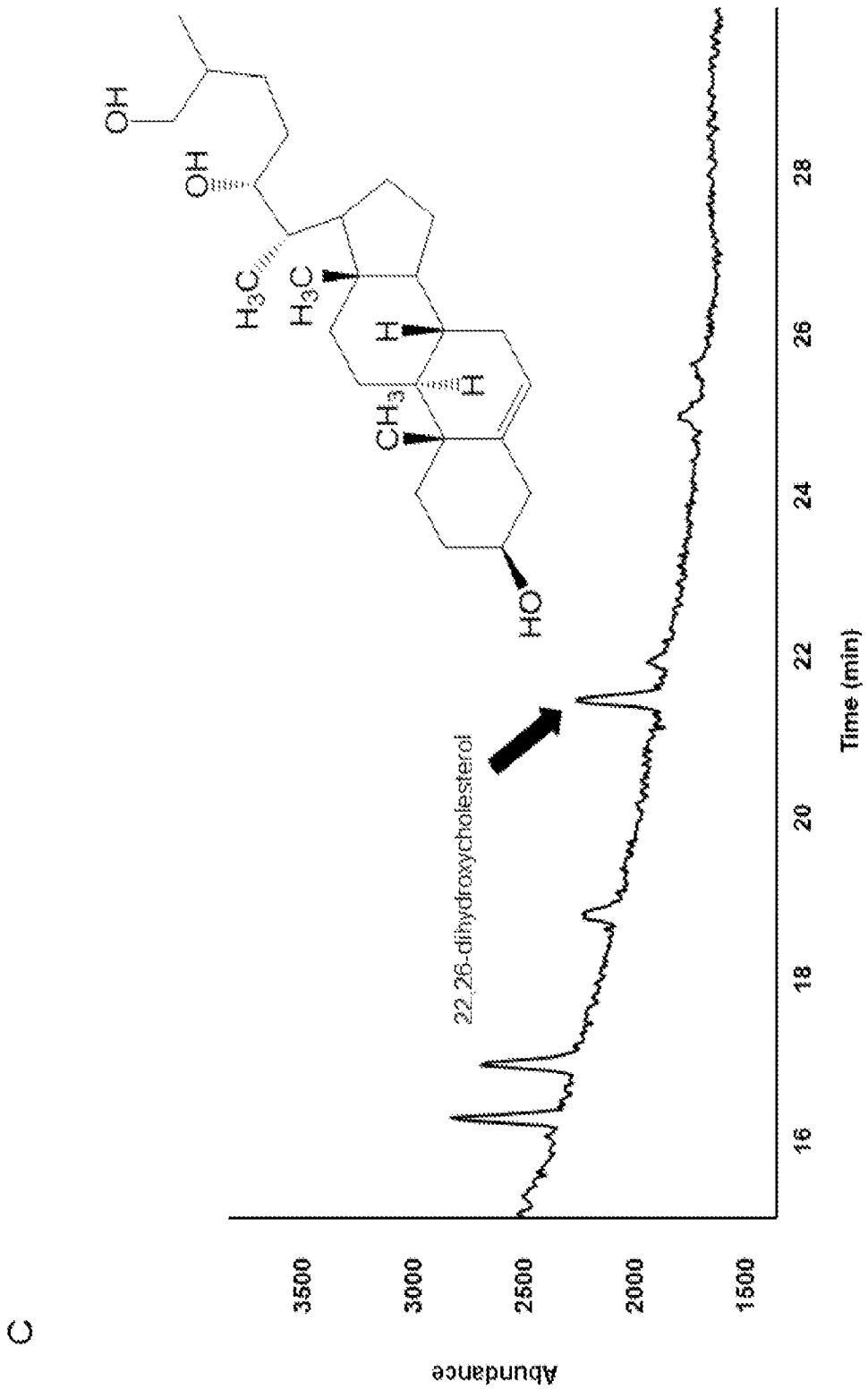

C
Abundance
3500
3000
2500
2000
1500
16
18
20
22
24
26
28
Time (min)
22,26-dihydroxycholesterol
OH
OH
H3C
H3C
H
H
CH3
HO

Figure 16C

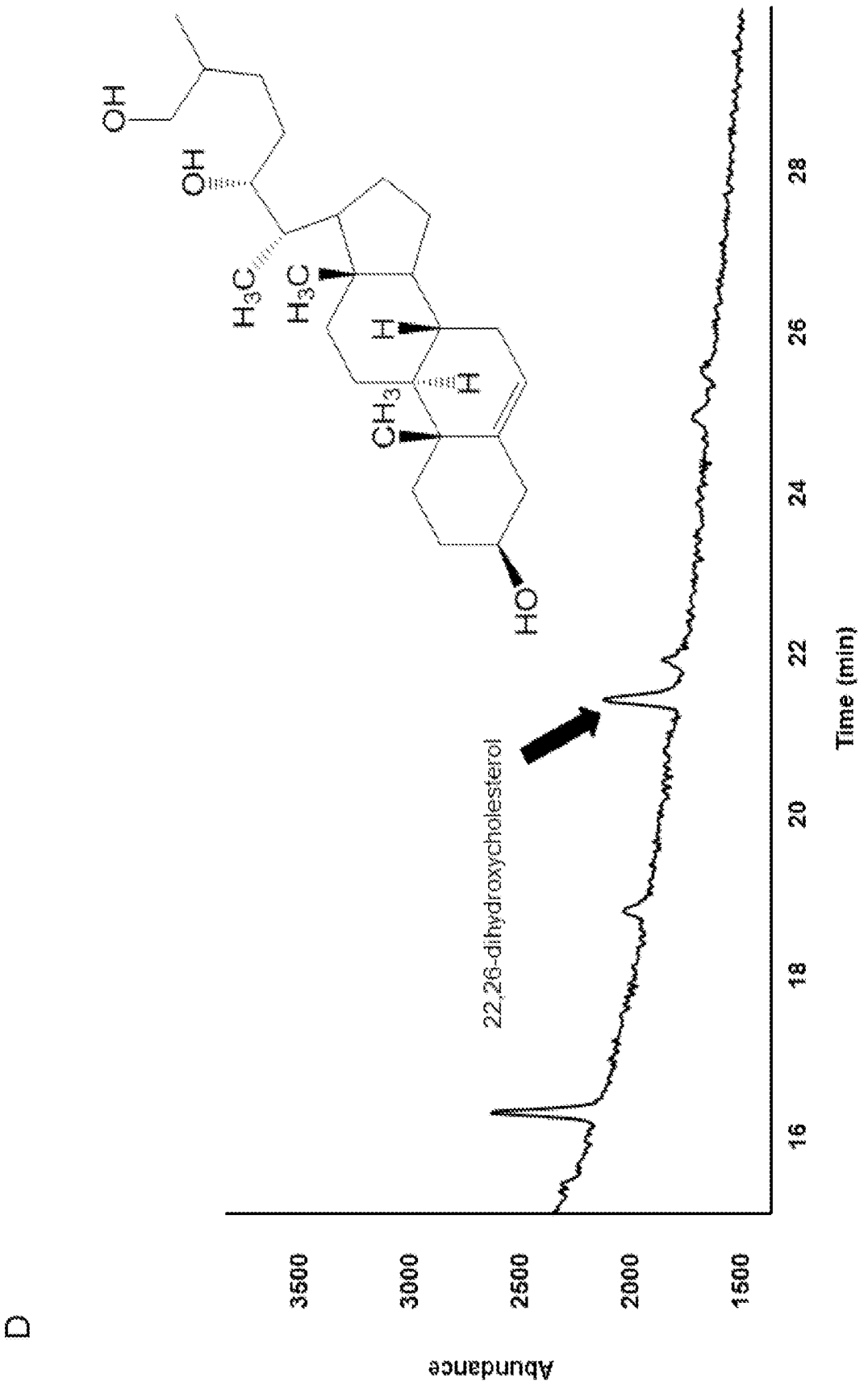

D
Abundance
3500
3000
2500
2000
1500
16
18
20
22
24
26
28
Time (min)
22,26-dihydroxycholesterol
HO
OH
OH
H_3C
H_3C
CH_3
H
H

Figure 16D

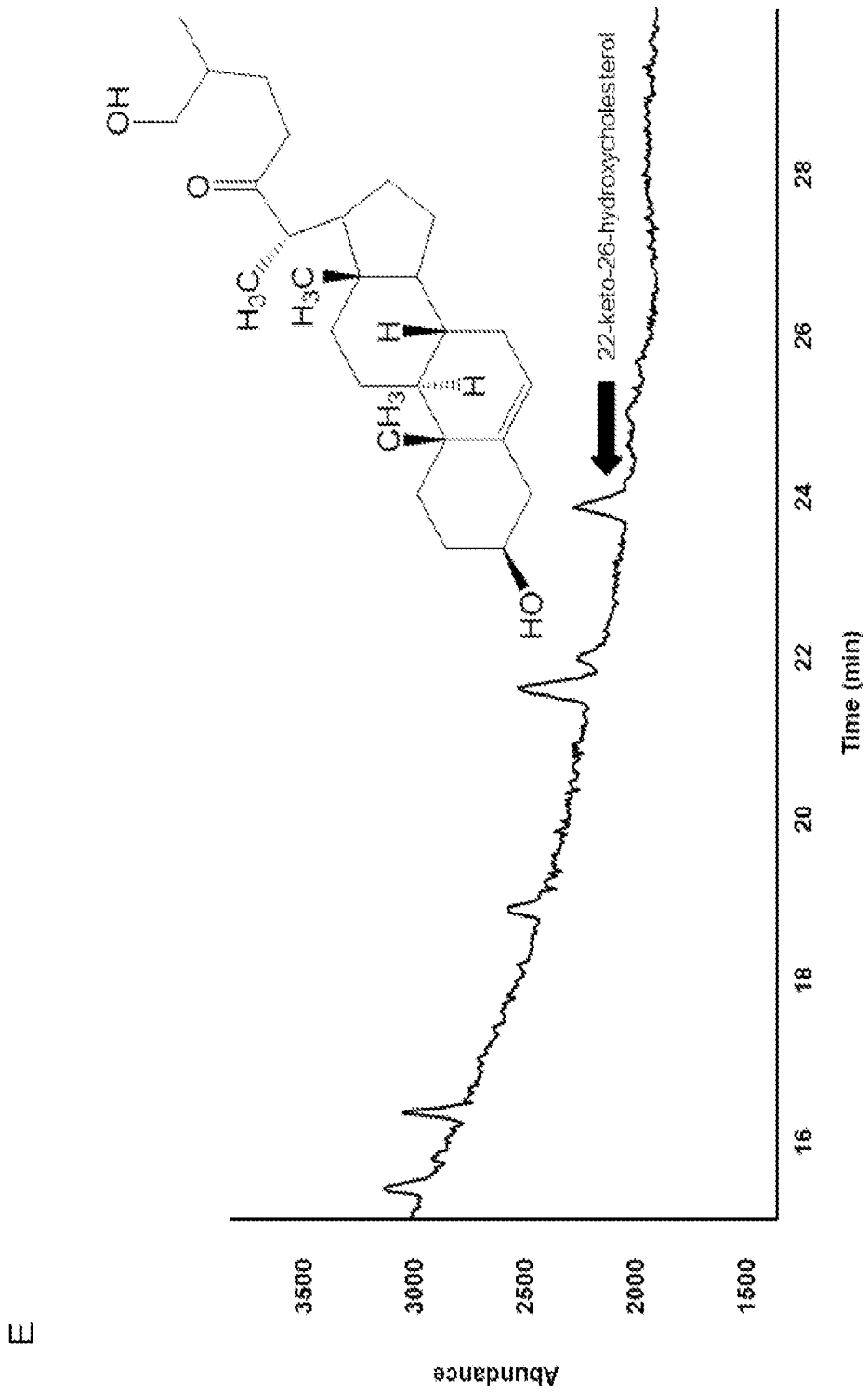

E
Abundance
3500
3000
2500
2000
1500
16
18
20
22
24
26
28
Time (min)
22-keto-26-hydroxycholesterol
OH
O
H3C
H3C
CH3
H
H
HO

Figure 16E

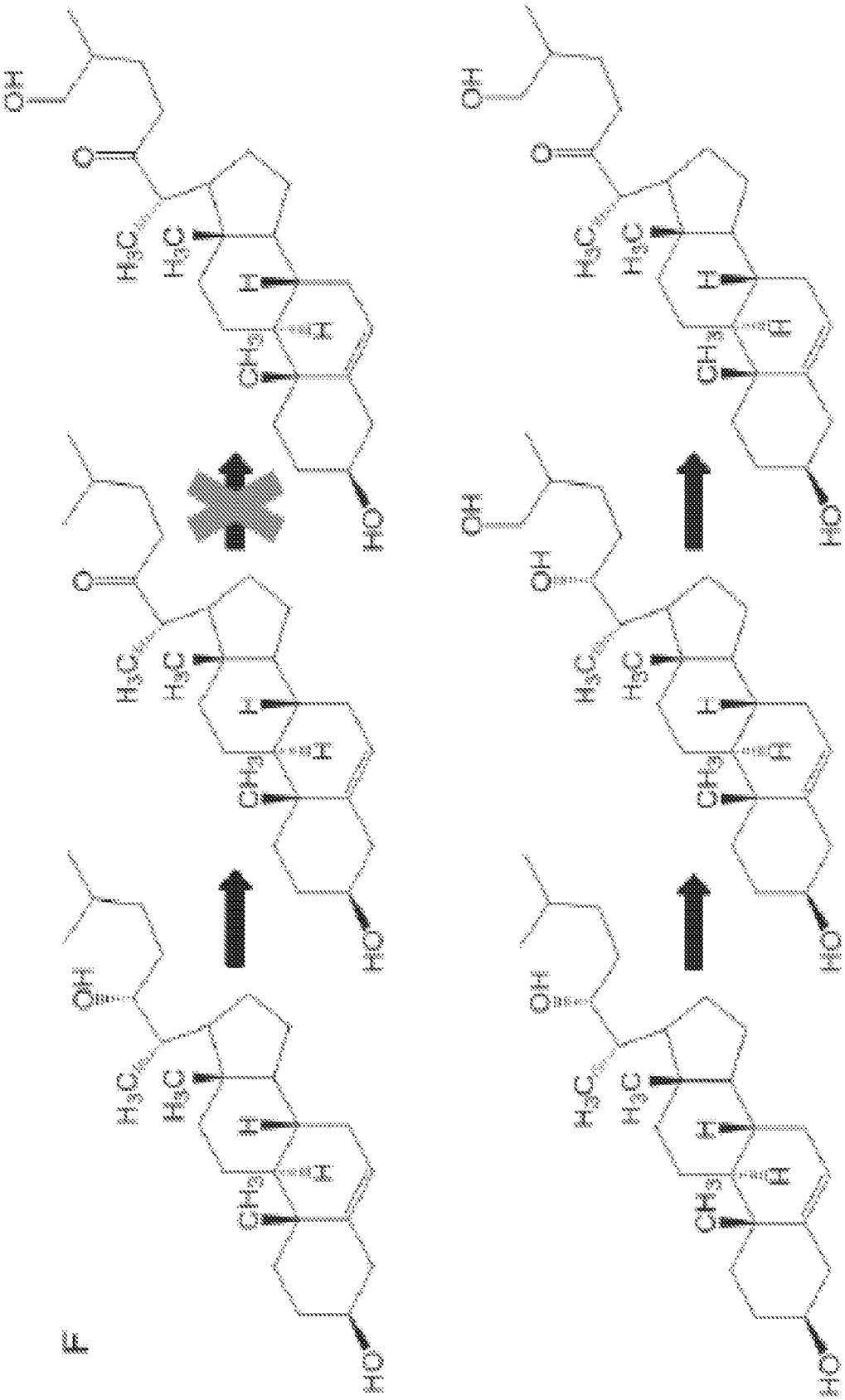


Figure 16F

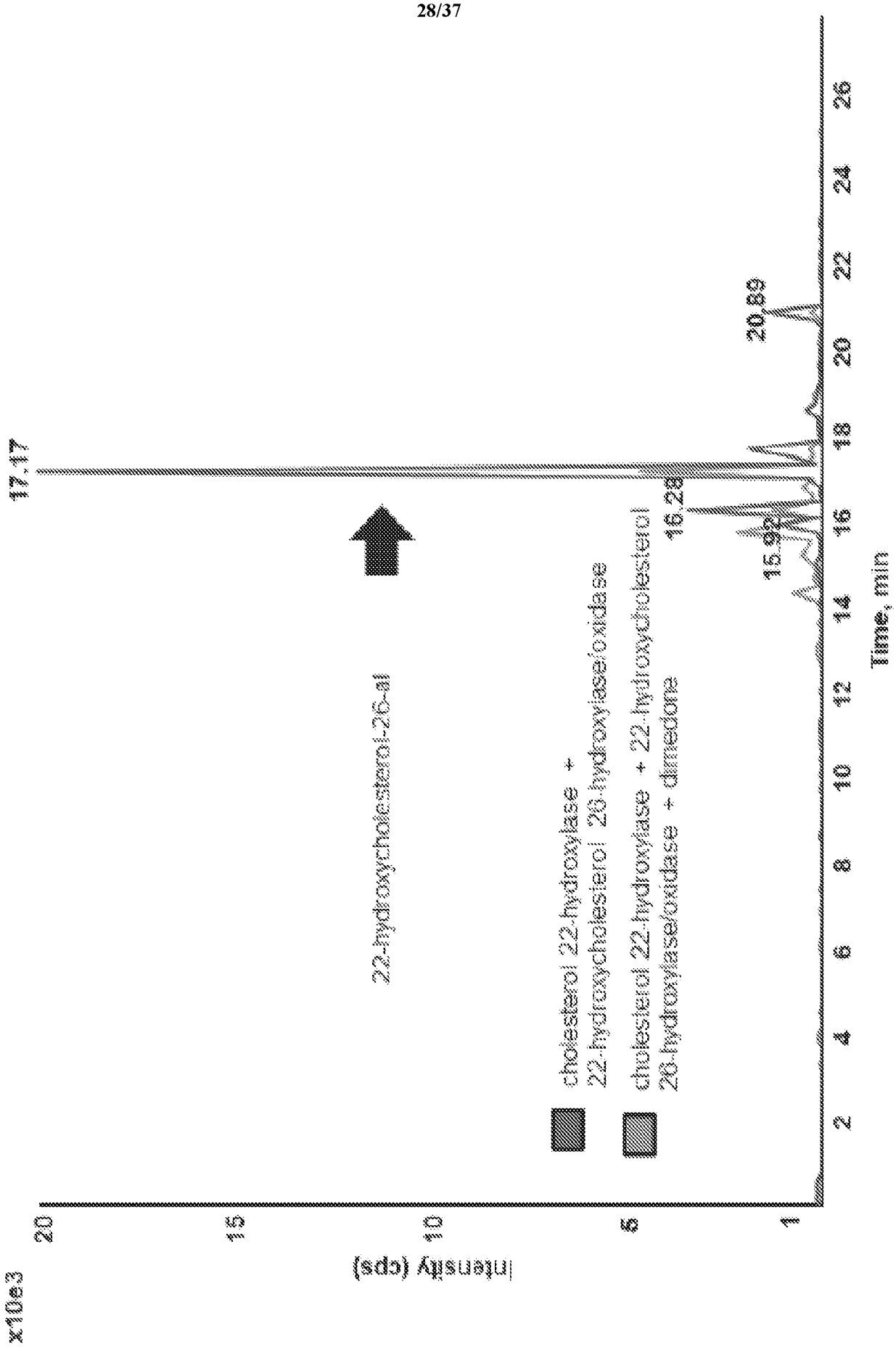

x10e3
20
15
10
5
1
Intensity (cps)
2
4
6
8
10
12
14
16
18
20
22
24
26
Time, min
17.17
16.28
15.92
20.89
22-hydroxycholestarol-26-al
cholesterol 22-hydroxylase +
22-hydroxycholesterol 20-hydroxylase/oxidase
cholesterol 22-hydroxylase + 22-hydroxycholesterol
20-hydroxylase/oxidase + dimedone

Figure 17

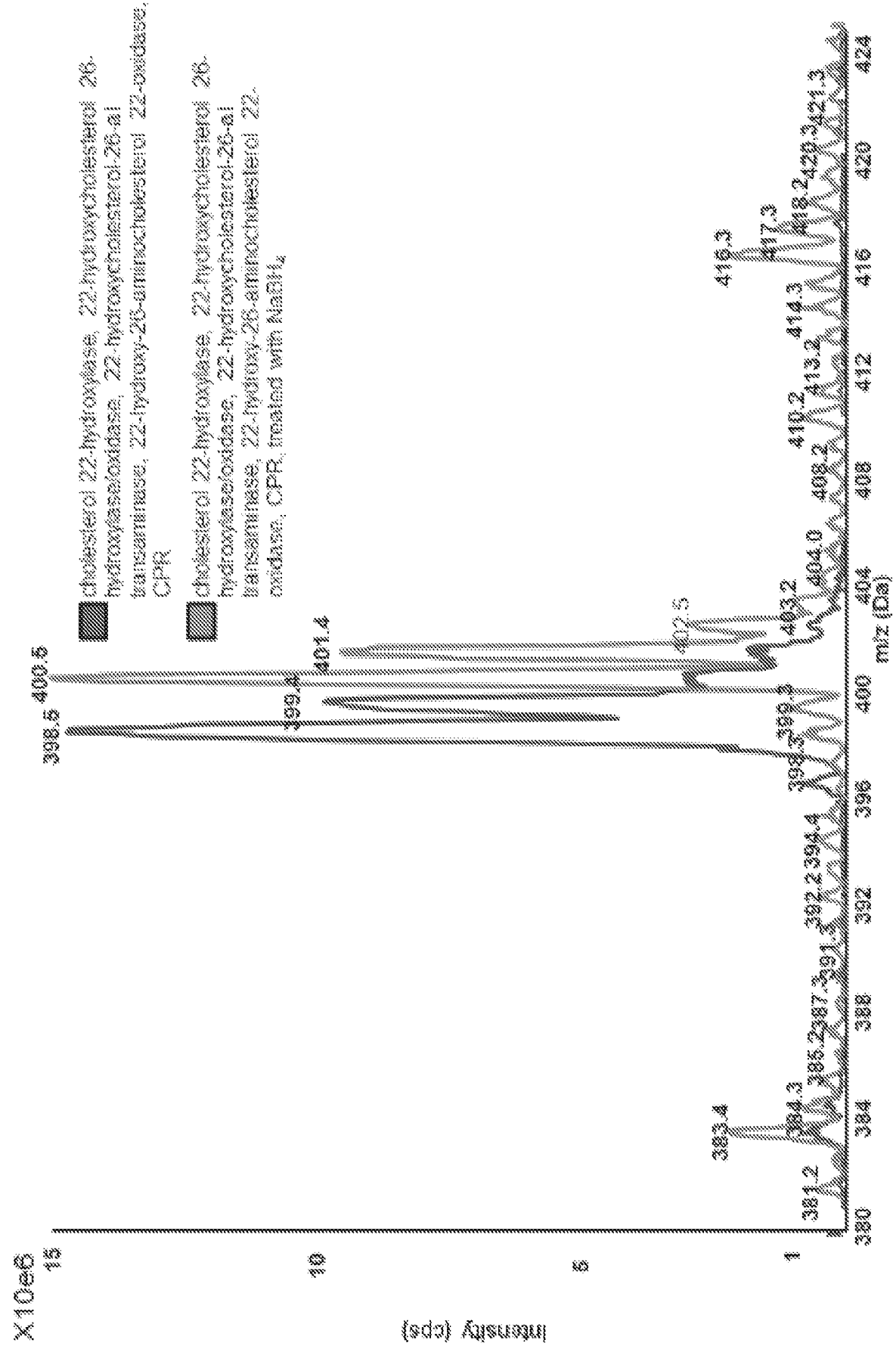

cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxycholesterol-26-al transaminase, 22-hydroxy-26-aminocholesterol 22-oxidase, CPR
cholesterol 22-hydroxylase, 22-hydroxycholesterol 26-hydroxylase/oxidase, 22-hydroxycholesterol-26-al transaminase, 22-hydroxy-26-aminocholesterol 22-oxidase, CPR, treated with NaBH4
X10e6
15
10
5
1
Intensity (cps)
380
384
388
392
396
400
404
408
412
416
420
424
m/z (Da)
381.2
383.4
384.3
385.2
387.3
391.3
392.2
394.4
398.3
398.5
399.3
399.4
400.6
401.4
402.5
403.2
404.0
406.2
410.2
413.2
414.3
416.3
417.3
418.2
420.3
421.3

Figure 18

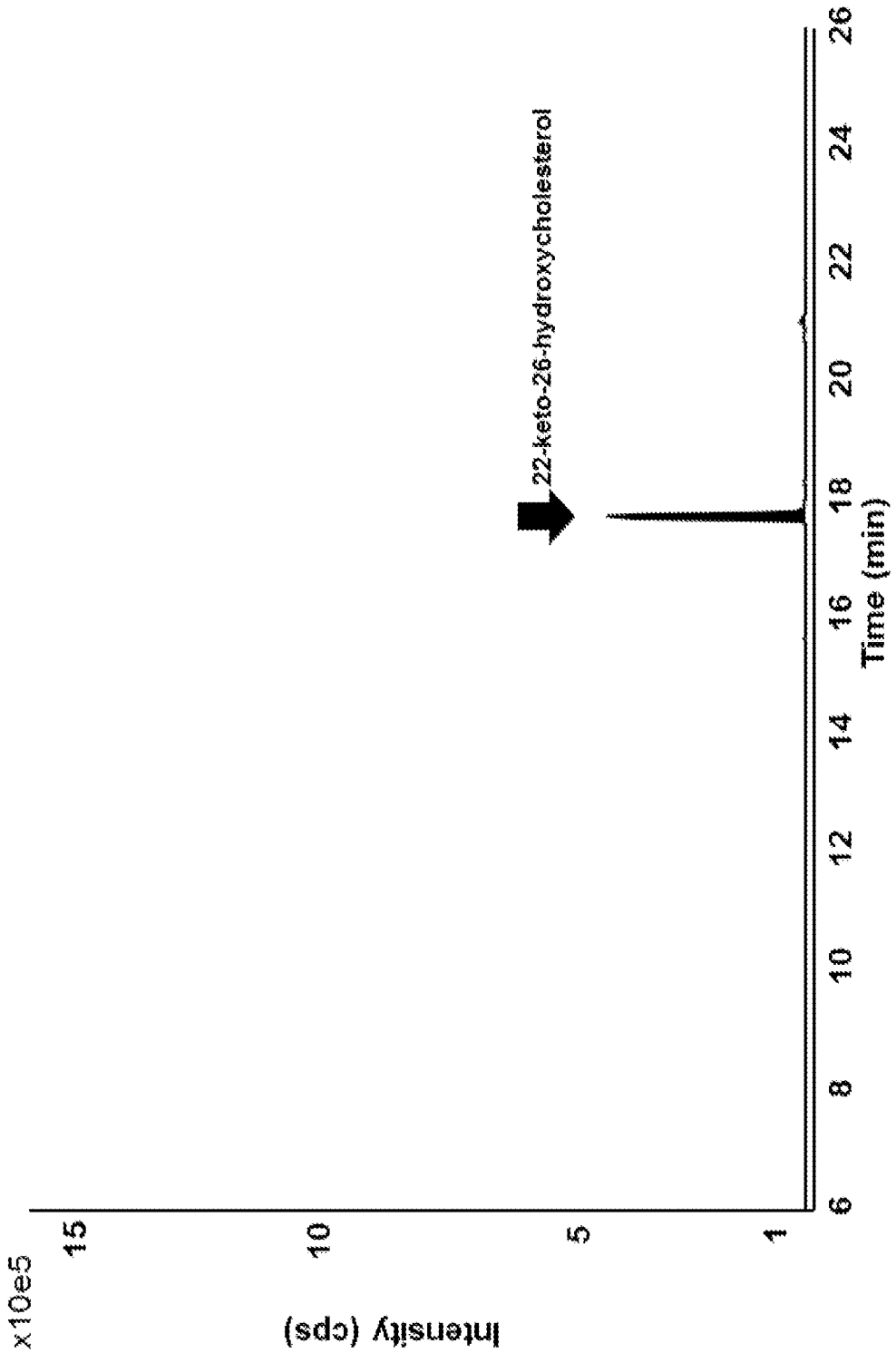

22-keto-26-hydroxycholesterol
Intensity (cps)
x10e5
15
10
5
1
6
8
10
12
14
16
18
20
22
24
26
Time (min)

Figure 19A

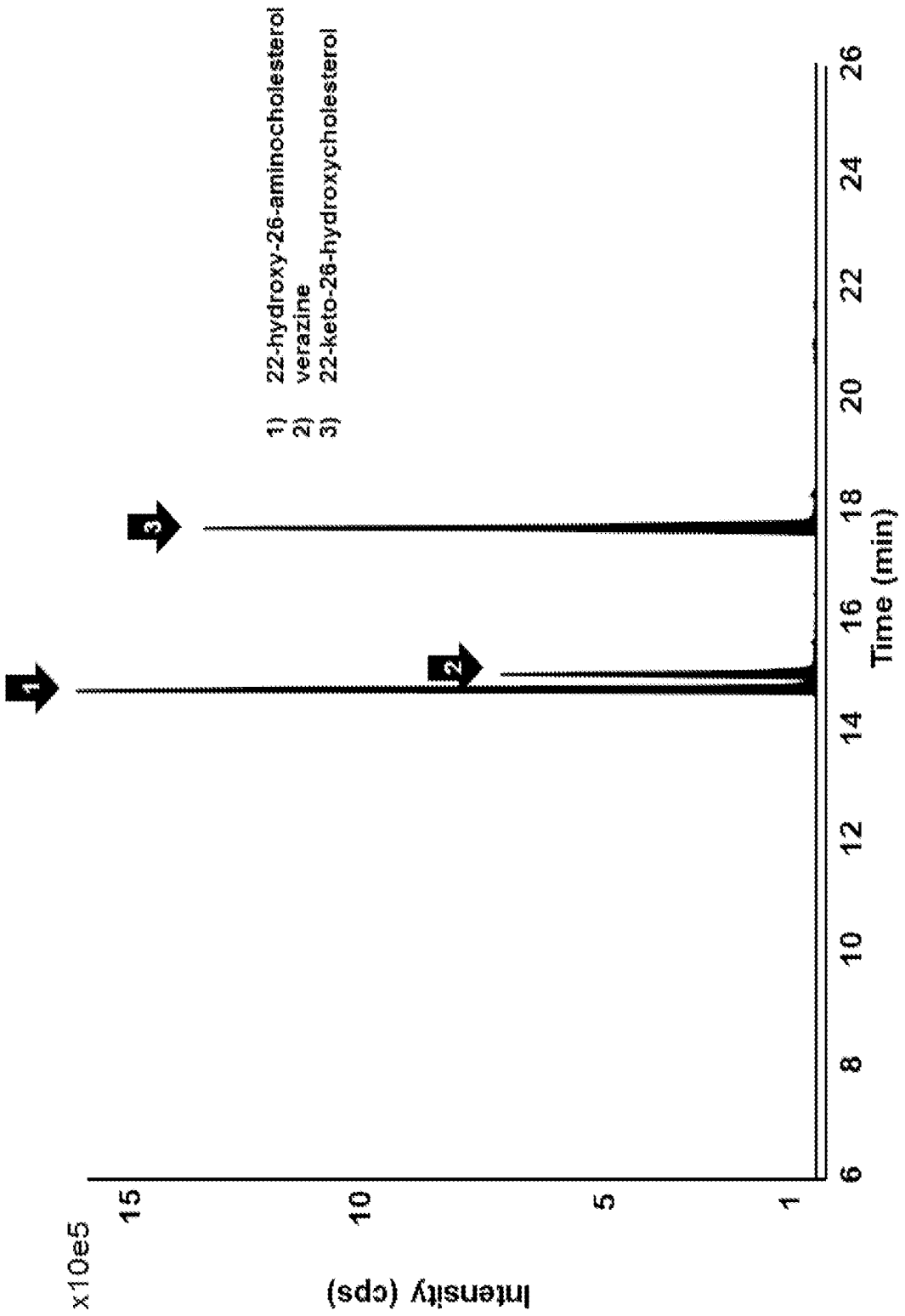

1) 22-hydroxy-26-aminocholesterol
2) verazine
3) 22-keto-26-hydroxycholesterol
1
2
3
x10e5
15
10
5
1
Intensity (cps)
6
8
10
12
14
16
18
20
22
24
26
Time (min)

Figure 19B

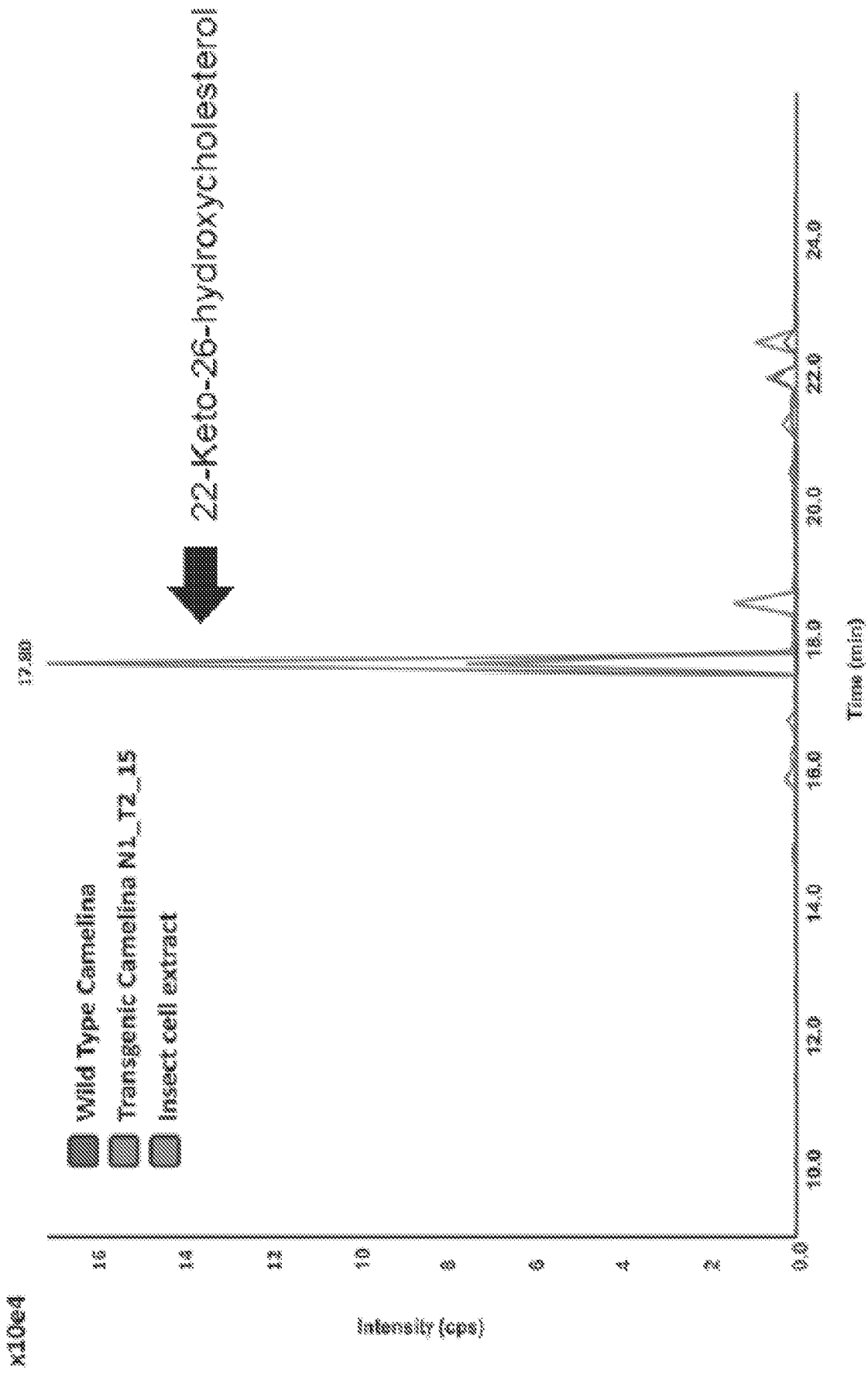

22-Keto-26-hydroxycholesterol
17.988
Wild Type Camelina
Transgenic Camelina M1_T2_15
Insect cell extract
x10e4
16
14
12
10
8
6
4
2
0.0
Intensity (cps)
10.0
12.0
14.0
16.0
18.0
20.0
22.0
24.0
Time (min)

Figure 20

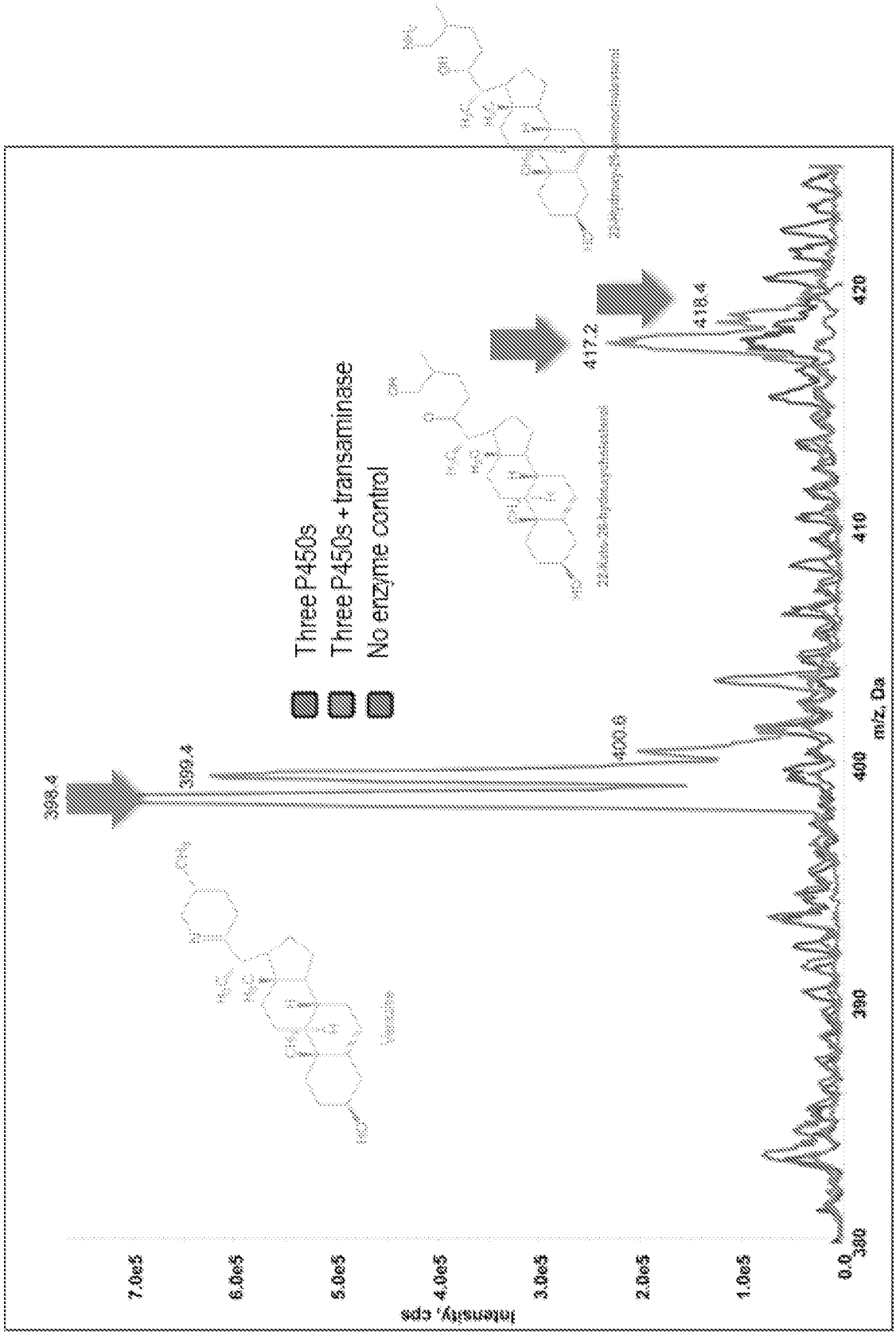

Three P450s
Three P450s + transaminase
No enzyme control
368.4
389.4
400.6
417.2
418.4
Intensity, cps
m/z, Da
7.0e5
6.0e5
5.0e5
4.0e5
3.0e5
2.0e5
1.0e5
0.0
380
390
400
410
420

Figure 21

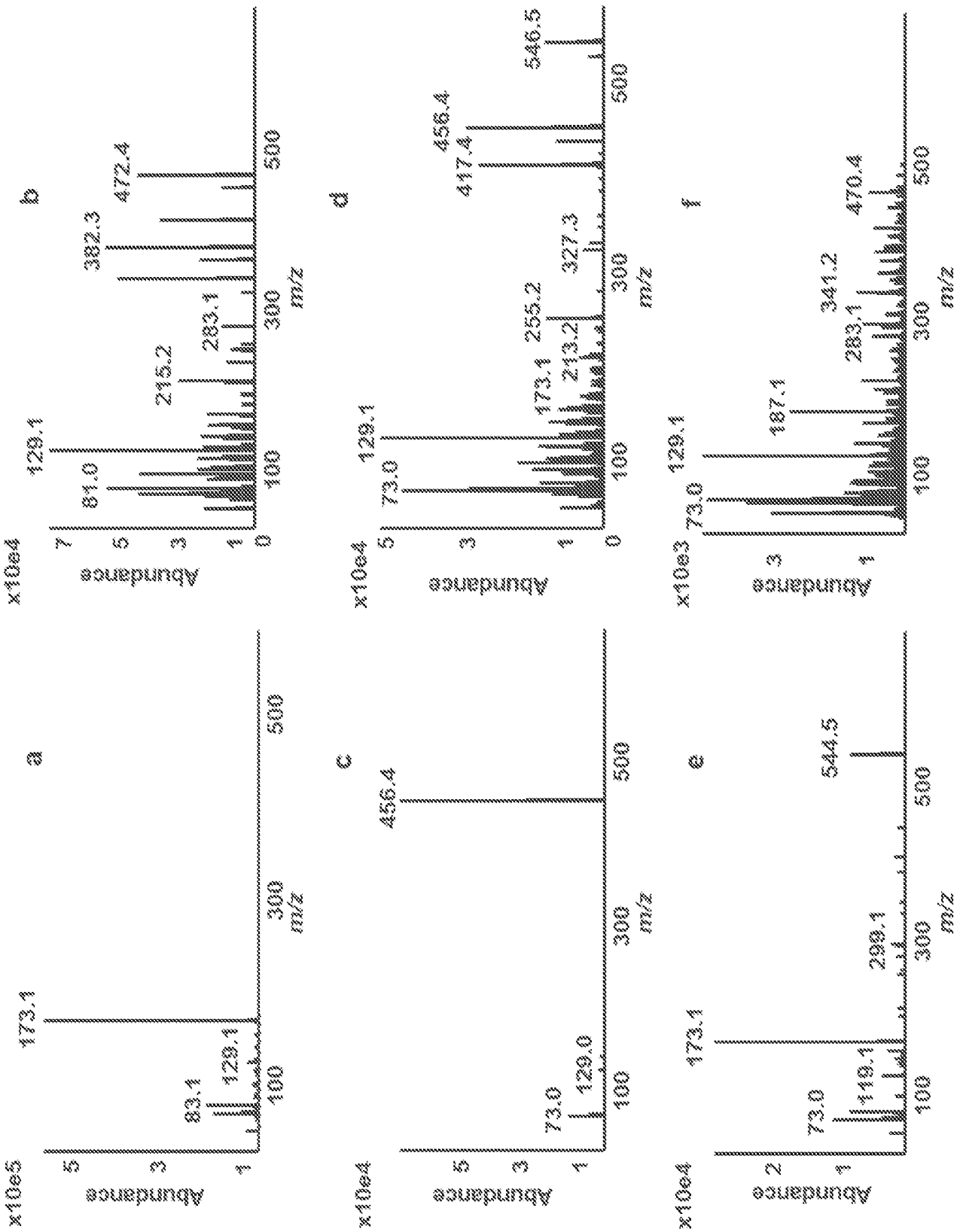

a
b
c
d
e
f
Abundance
m/z
x10e5
x10e4
x10e3
173.1
129.1
83.1
81.0
215.2
283.1
382.3
472.4
456.4
73.0
129.0
255.2
213.2
327.3
417.4
546.5
544.5
299.1
118.1
187.1
341.2
470.4

Figure 22

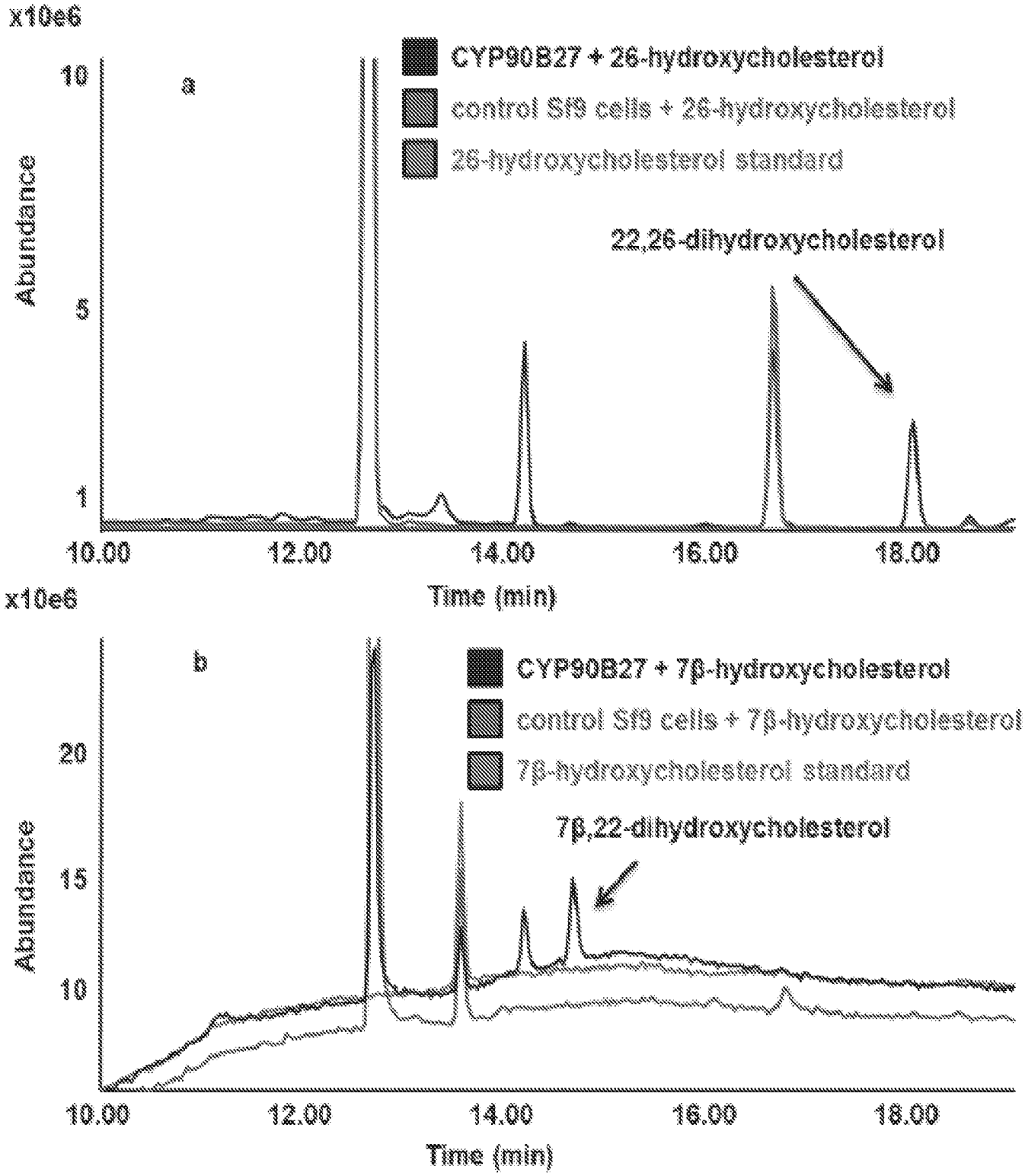

x10e6
10
5
1
Abundance
a
CYP90B27 + 26-hydroxycholesterol
control Sf9 cells + 26-hydroxycholesterol
26-hydroxycholesterol standard
22,26-dihydroxycholesterol
10.00
12.00
14.00
16.00
18.00
Time (min)
x10e6
20
15
10
Abundance
b
CYP90B27 + 7β-hydroxycholesterol
control Sf9 cells + 7β-hydroxycholesterol
7β-hydroxycholesterol standard
7β,22-dihydroxycholesterol
10.00
12.00
14.00
16.00
18.00
Time (min)

Figure 23

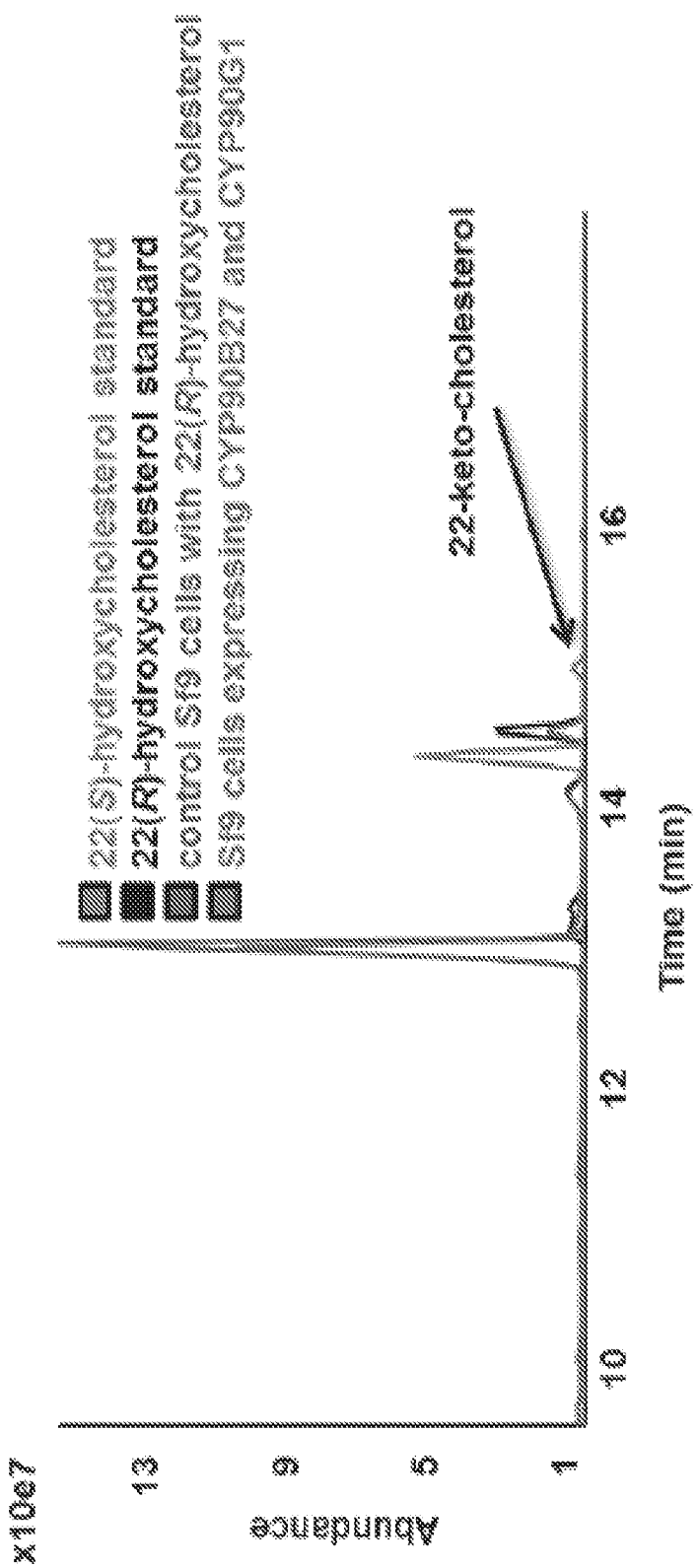

22(S)-hydroxycholesterol standard
22(R)-hydroxycholesterol standard
control Sf9 cells with 22(R)-hydroxycholesterol
Sf9 cells expressing CYP90B27 and CYP90C1
22-keto-cholesterol
x10e7
13
9
5
1
Abundance
10
12
14
16
Time (min)

Figure 24

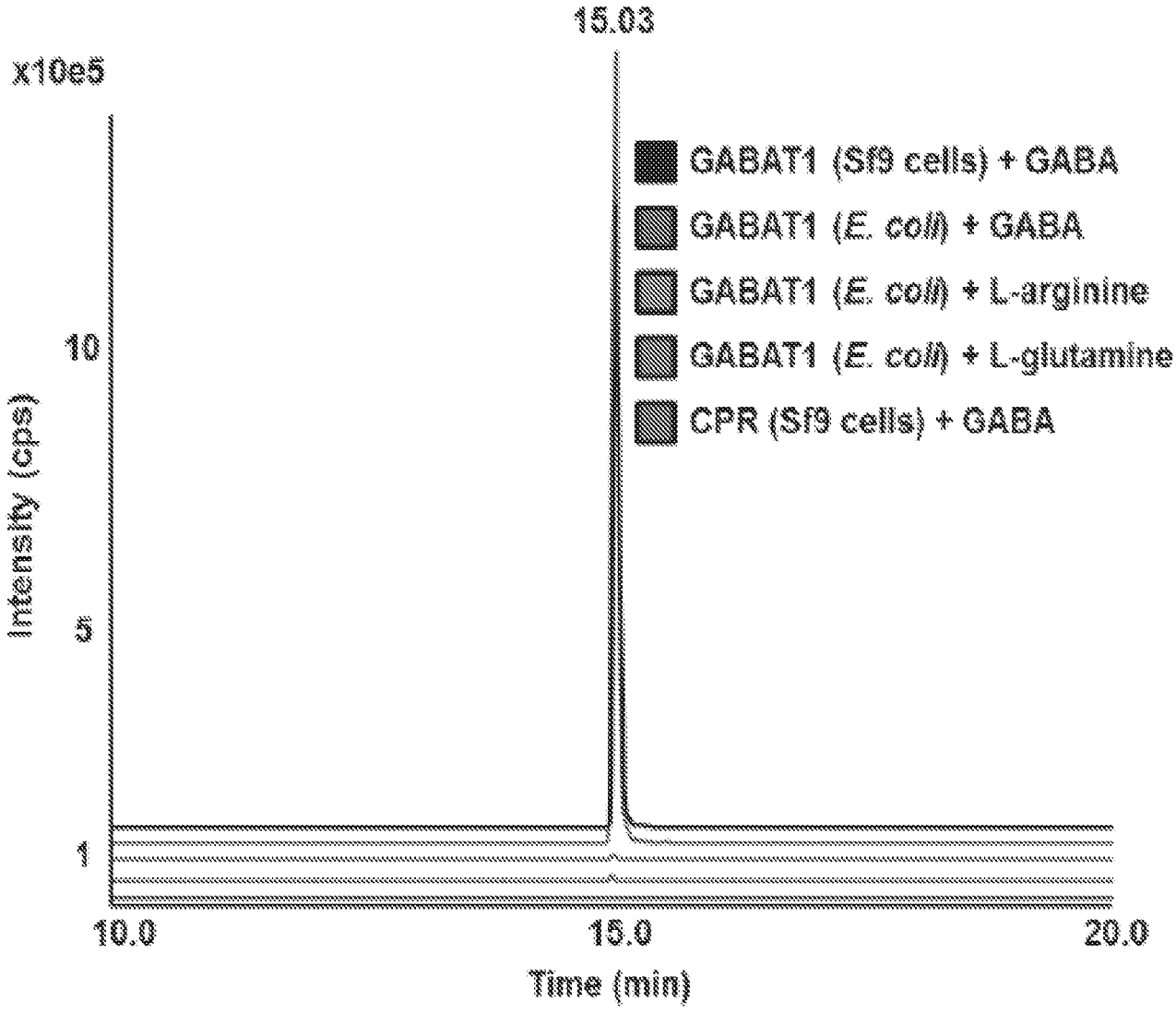

15.03
x10e5
GABAT1 (Sf9 cells) + GABA
GABAT1 (E. coli) + GABA
GABAT1 (E. coli) + L-arginine
GABAT1 (E. coli) + L-glutamine
CPR (Sf9 cells) + GABA
10
5
1
Intensity (cps)
10.0
15.0
20.0
Time (min)

Figure 25