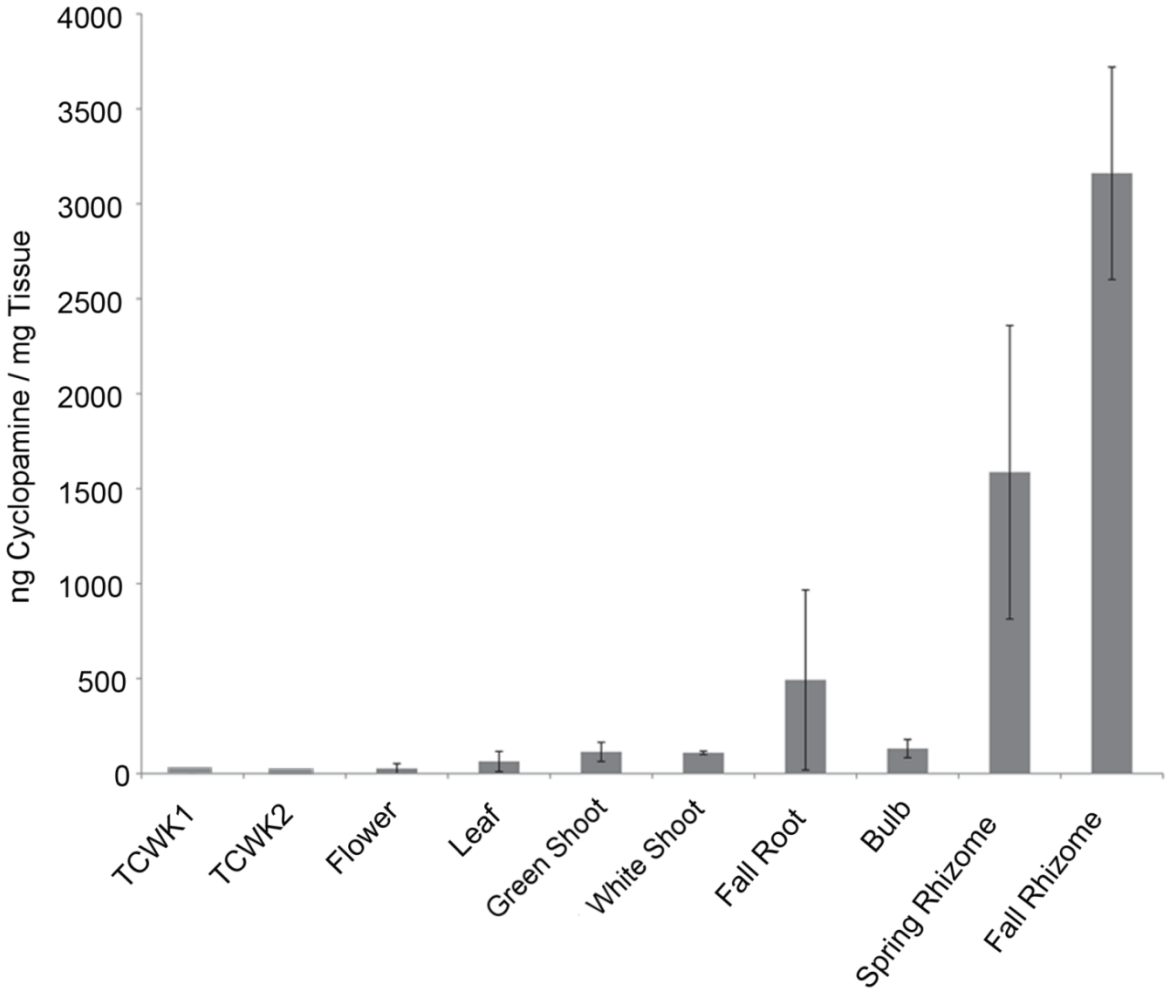


Figure S1. Cyclopamine accumulation profile in *Veratrum californicum.* Each *V. californicum* tissue underwent three independent ethanol extractions followed by LC-MS/MS analysis on a 4000 QTRAP. Quantitation with authentic cyclopamine was accomplished using a standard curve with peak areas. Each value represents ng of alkaloid per mg of tissue; error bars represent standard deviation. Sample dilutions were as follows: 10-fold for tissue culture samples, 1,000-fold for flower, 5,000-fold for leaf and shoot, and 10,000-fold for root, bulb, and rhizome. TCWK1 and TCWK2 stand for tissue culture one- and two weeks after transfer to new media, respectively.

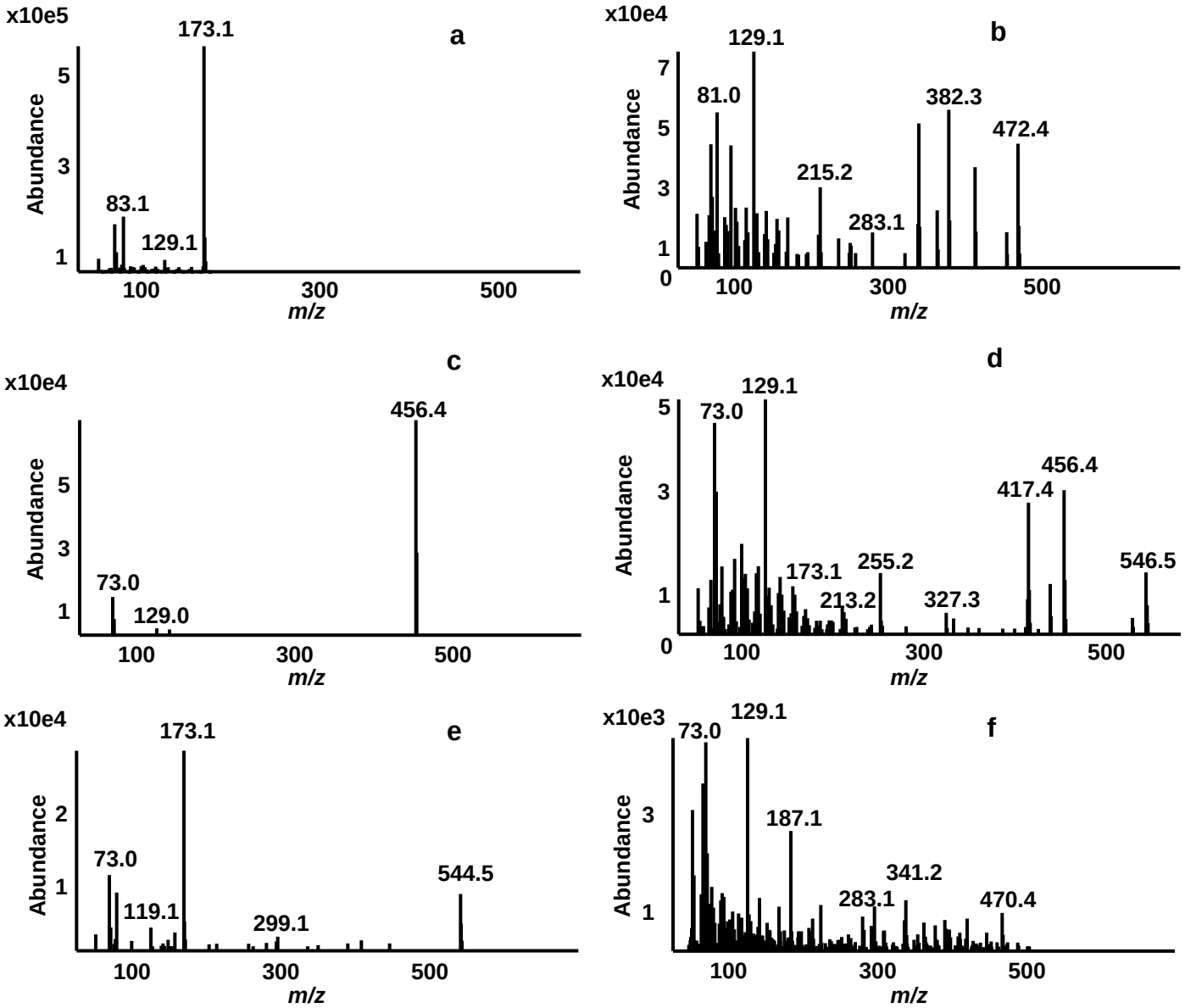


Figure S2. Mass spectra of select derivatized standards and enzymatically formed products. Enzyme assays using recombinant *Veratrum californicum* genes and authentic standards were first extracted with hexane and derivatized with Sylon HTP before GC-MS analysis. (a) Spectrum of 22(*R*)-hydroxycholesterol 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.

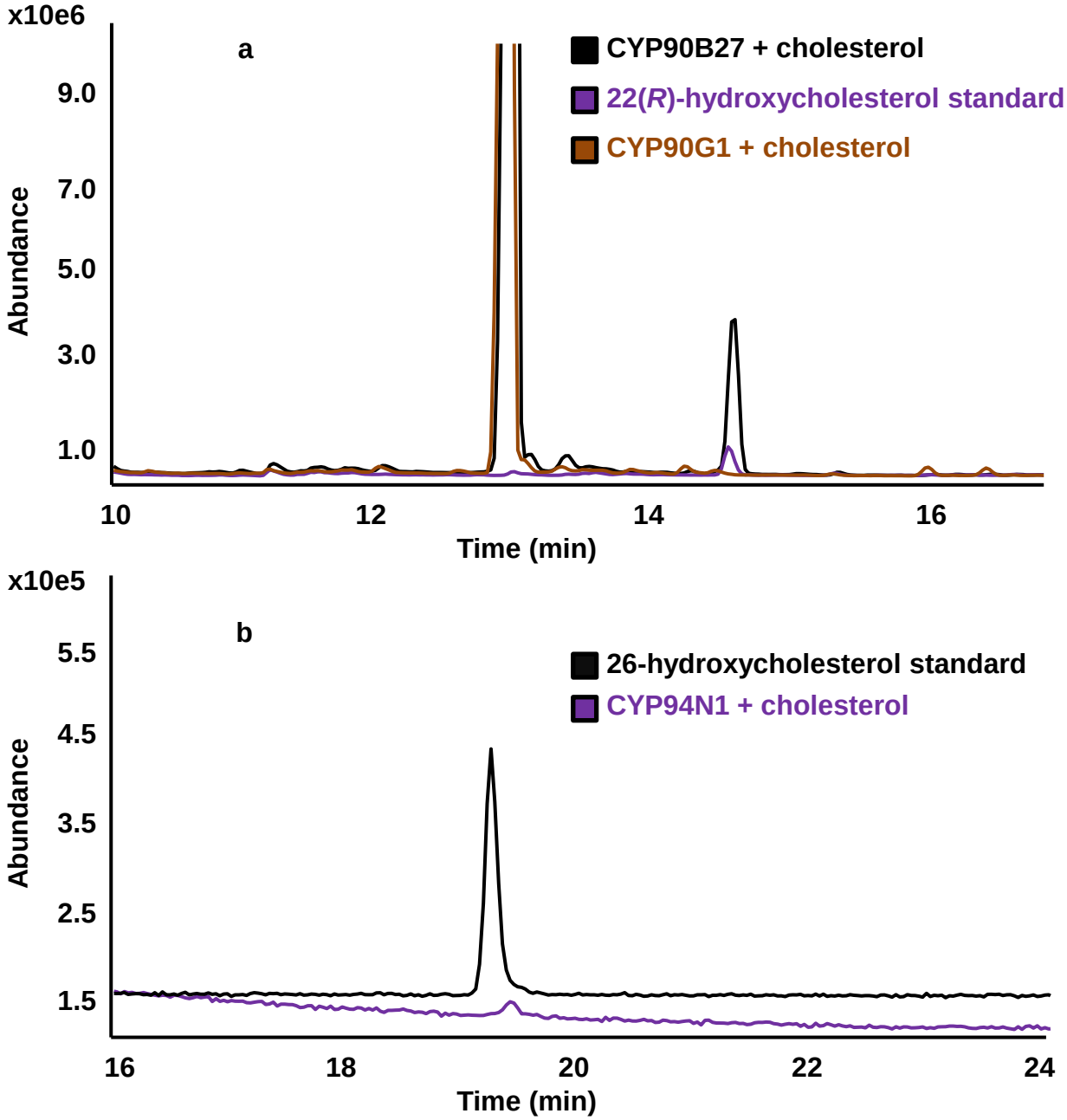


Figure S3. GC-MS analysis of selected *Veratrum californicum* cytochrome P450 enzyme assays with cholesterol. Enzyme assays using *S. frugiperda* Sf9 cells expressing each enzyme were extracted and derivatized with Sylon HTP before GC-MS analysis. (a) Overlay of enzyme assays performed with either CYP90B27 (cholesterol 22-hydroxylase) or CYP90G1 (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 CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase) co-expressed with CPR and cholesterol as substrate, overlaid with pure 26-hydroxycholesterol [27(25R)-hydroxycholesterol] as reference.

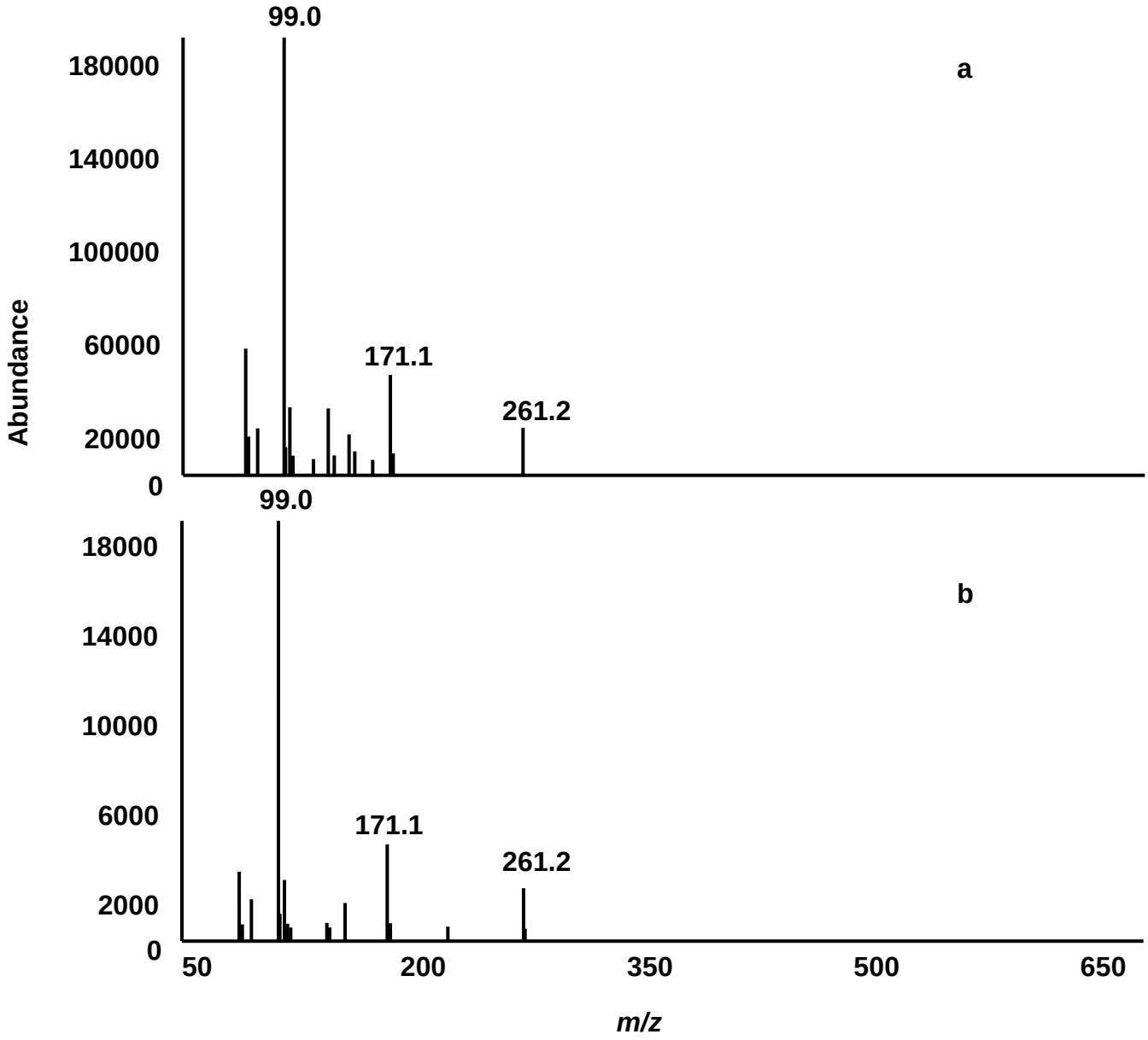


Figure S4. Mass spectra of enzymatically formed 22,26-dihydroxycholesterol. Mass spectrum of product formed by enzyme assays using (a) *S. frugiperda* Sf9 cells expressing *Veratrum californicum* CYP90B27 (cholesterol 22-hydroxylase) and *E. californica* cytochrome P450 reductase (CPR) with pure 26-hydroxycholesterol [27(25*R*)-hydroxycholesterol] as substrate and (b) CYP90B27, *V. californicum* CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase), and CPR with pure cholesterol as substrate were extracted and derivatized with Sylon HTP before GC-MS analysis. Mass spectra after background subtraction is shown.

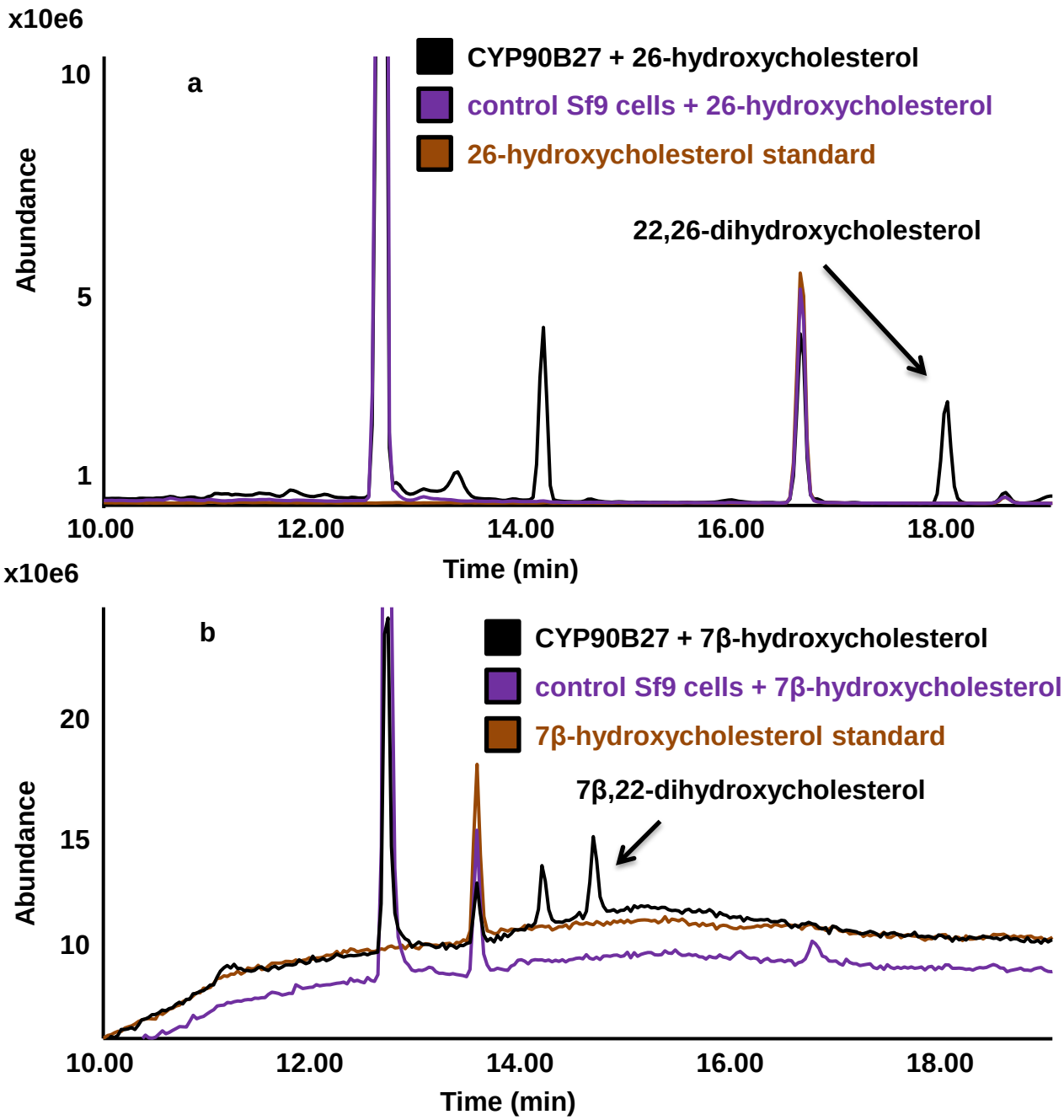


Figure S5. GC-MS analysis of *Veratrum californicum* CYP90B27 with 26-hydroxycholesterol and 7β-hydroxycholesterol. Enzyme assays using *S. frugiperda* Sf9 cells expressing CYP90B27 (cholesterol 22-hydroxylase) and *E. californica* cytochrome P450 reductase (CPR) were extracted and derivatized with Sylon 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.

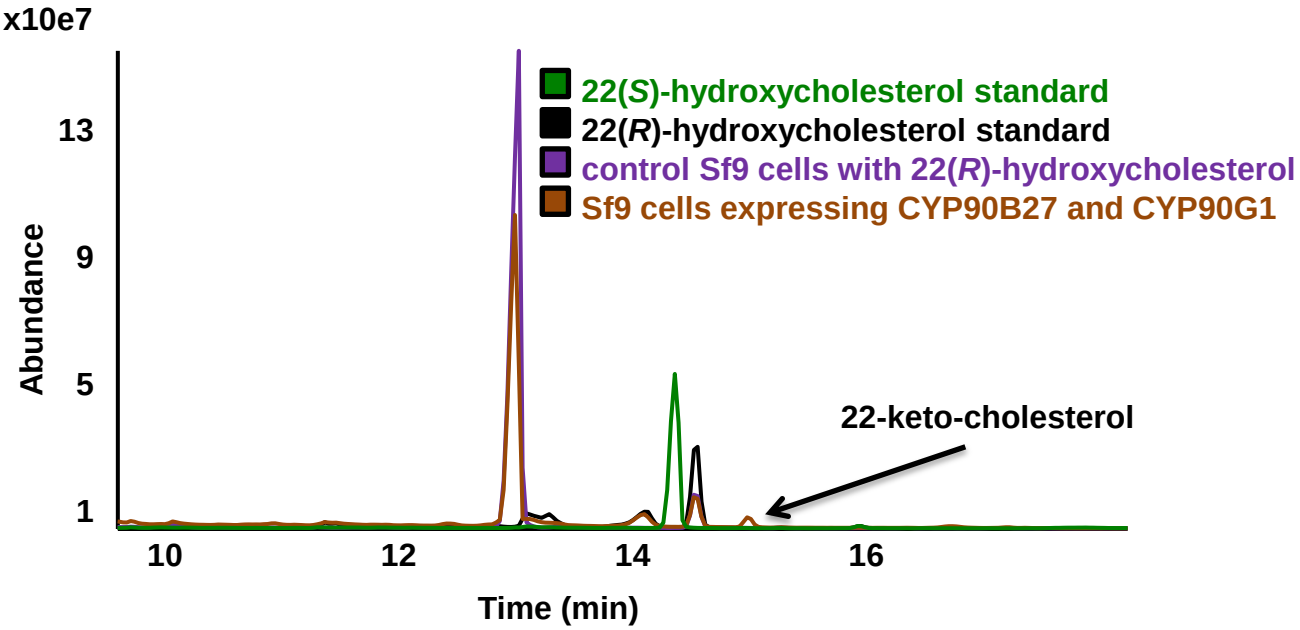
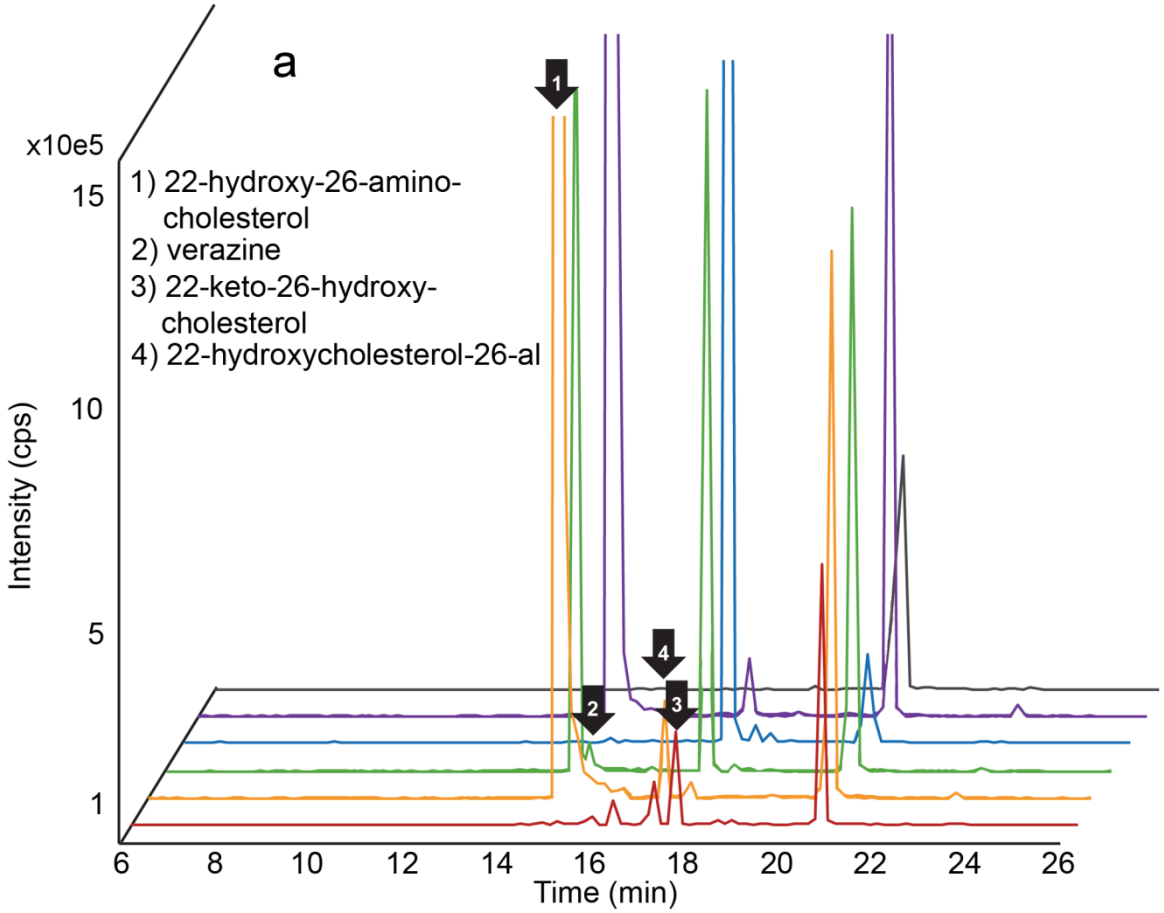


Figure S6. **GC-MS analysis of *Veratrum californicum* cytochrome P450 enzymes CYP90B27 and CYP90G1.** *S. frugiperda* Sf9 cells expressing enzyme CYP90B27 (cholesterol 22-hydroxylase), CYP90G1 (22-hydroxy-26-aminocholesterol 22-oxidase), and *E. californica* cytochrome P450 reductase (CPR) were extracted 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.


a
x10e5
15
10
5
1
Intensity (cps)
1) 22-hydroxy-26-amino-cholesterol
2) verazine
3) 22-keto-26-hydroxy-cholesterol
4) 22-hydroxycholesterol-26-al
1
2
3
4
6
8
10
12
14
16
18
20
22
24
26
Time (min)

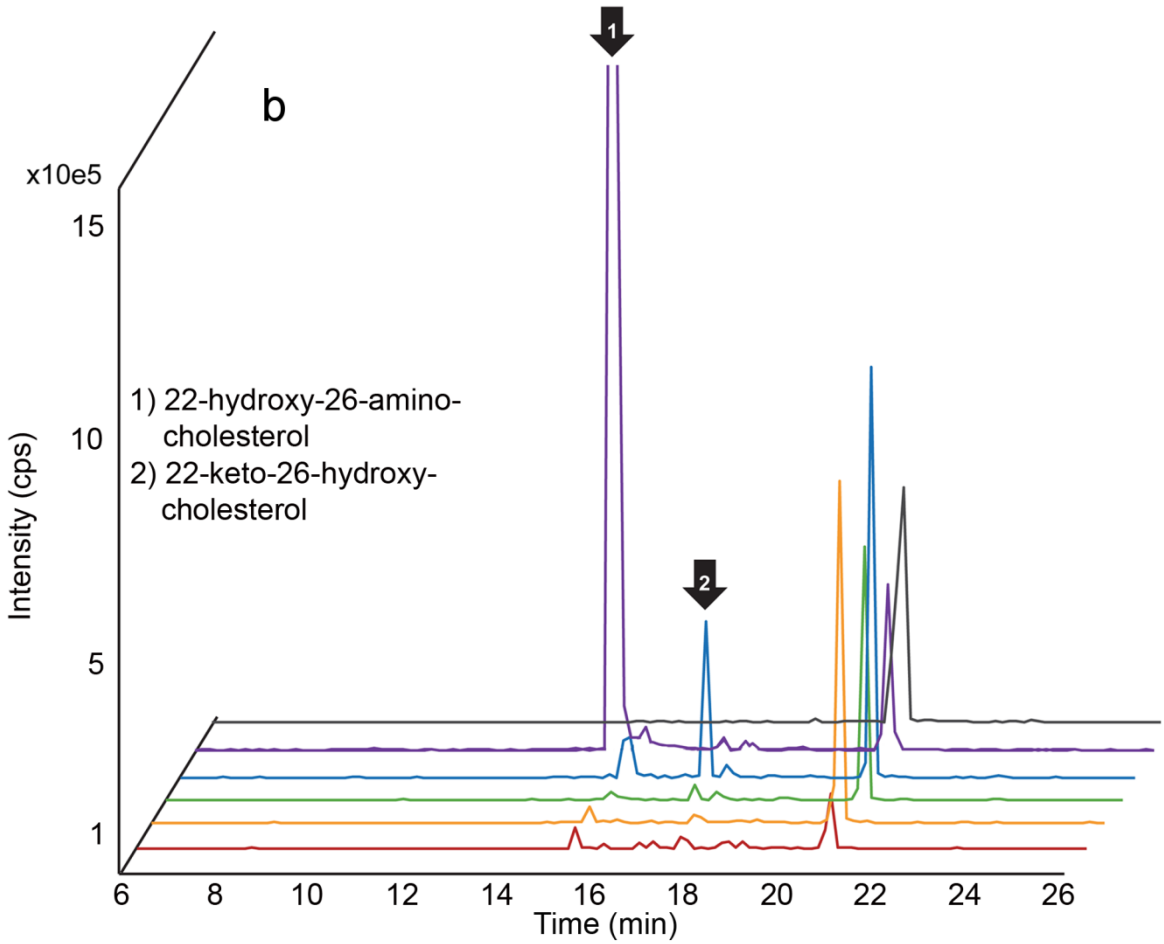

b
x10e5
15
10
5
1
Intensity (cps)
1) 22-hydroxy-26-amino-cholesterol
2) 22-keto-26-hydroxy-cholesterol
1
2
6
8
10
12
14
16
18
20
22
24
26
Time (min)

Figure S7. Determination of the order of the enzymatic steps to verazine. Enzyme assays were performed as described in Figure S11 b using crude *S. frugiperda* Sf9 cells infected with baculovirus containing each *Veratrum californicum* gene. All cytochromes P450 were co-expressed with *E. californica* cytochrome P450 reductase (CPR); GABAT1 (22-hydroxycholesterol-26-al transaminase) was expressed alone. Cholesterol substrate for each initial reaction was provided by Sf9 cells. Extracts were taken at each step and analyzed by LC-MS/MS. Chromatograms for each sample are a combination of product ions for clarity. (a) Chromatograms of assays run initially with CYP90B27 (cholesterol 22-hydroxylase) combined with CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase). Each colored chromatograph corresponds to the following: **Red**-CYP90B27 + CYP94N1, **Orange**-CYP90B27 + CYP94N1, extracted, then added as substrate to assay with GABAT1, **Green**-CYP90B27 + CYP94N1, extracted, then added as substrate to assay with GABAT1, extracted, and added as substrate to CYP90G1 (22-hydroxy-26-aminocholesterol 22-oxidase), **Blue**-CYP90B27 + CYP94N1 extracted and added as substrate to CYP90G1, **Purple**-CYP90B27 + CYP94N1, extracted, and added as substrate to CYP90G1, extracted, and added as substrate to GABAT1, **Grey**-CPR only control. (b) Chromatograms of assays run initially with CYP90B27 + CYP90G1. Each colored chromatogram corresponds to the following: **Red**-CYP90B27 + CYP90G1, **Orange**-CYP90B27 + CYP90G1, extracted, and added as substrate to GABAT1, **Green**-CYP90B27 + CYP90G1, extracted, and added as substrate to GABAT1, extracted, then added as substrate to CYP94N1, **Blue**-CYP90B27 + CYP90G1, extracted, and added as substrate to CYP94N1, **Purple**-CYP90B27 + CYP90G1, extracted, and added as substrate to CYP94N1, extracted, then added as substrate to GABAT1, **Grey**-CPR only control.

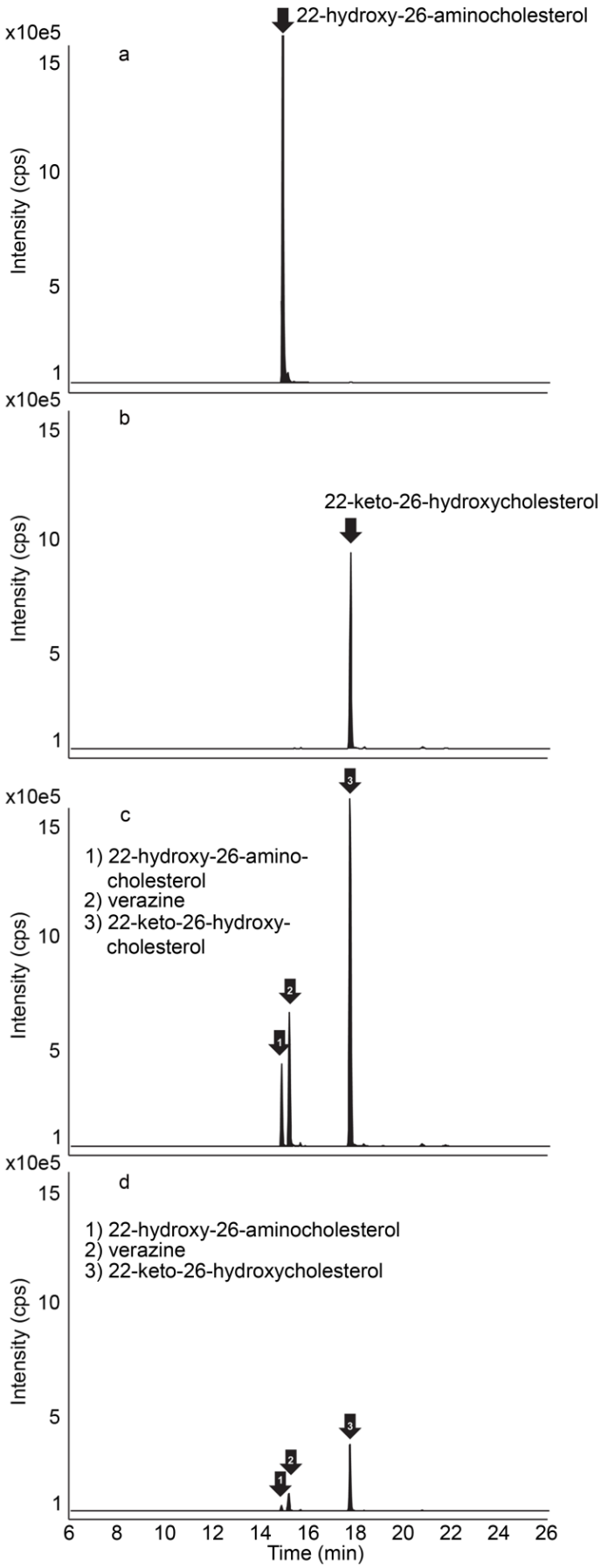


Figure S8. LC-MS/MS of *S. frugiperda* Sf9 extracts expressing *Veratrum californicum* genes. Sf9 cells infected with several combinations of baculovirus containing select genes from *V. californicum* were extracted and analyzed by LC-MS/MS. (a) Extract of Sf9 infection with CYP90B27 (cholesterol 22-hydroxylase), CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase), GABAT1 (22-hydroxycholesterol-26-al transaminase), and CPR, (b) extract of Sf9 infection with CYP90B27, CYP94N1, CYP90G1 (22-hydroxy-26-aminocholesterol 22-oxidase), and CPR, (c) extract of Sf9 infection with CYP90B27, CYP94N1, GABAT1, CYP90G1, and CPR, (d) extract of Sf9 infection with CYP90B27, CYP94N1, CYP90G1, *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.

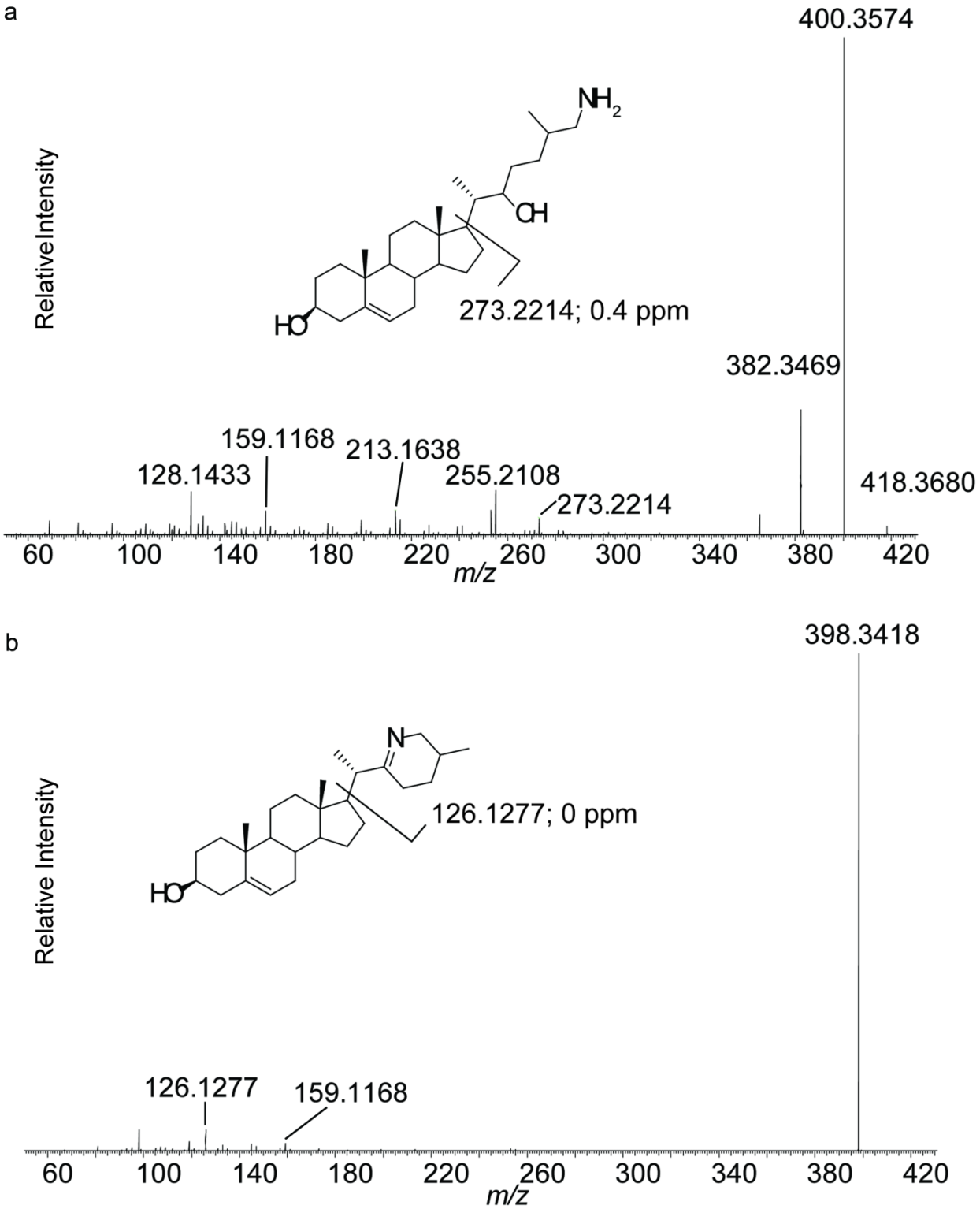


Figure S9. High-resolution mass spectrometric analysis of enzymatically formed 22-hydroxy-26-aminocholesterol and verazine. Purified (a) 22-hydroxy-26-aminocholesterol and (b) verazine were analyzed by high-resolution mass spectrometry with a LTQ-Orbitrap Velos Pro by direct infusion for structural identification. Key fragments for each are indicated.

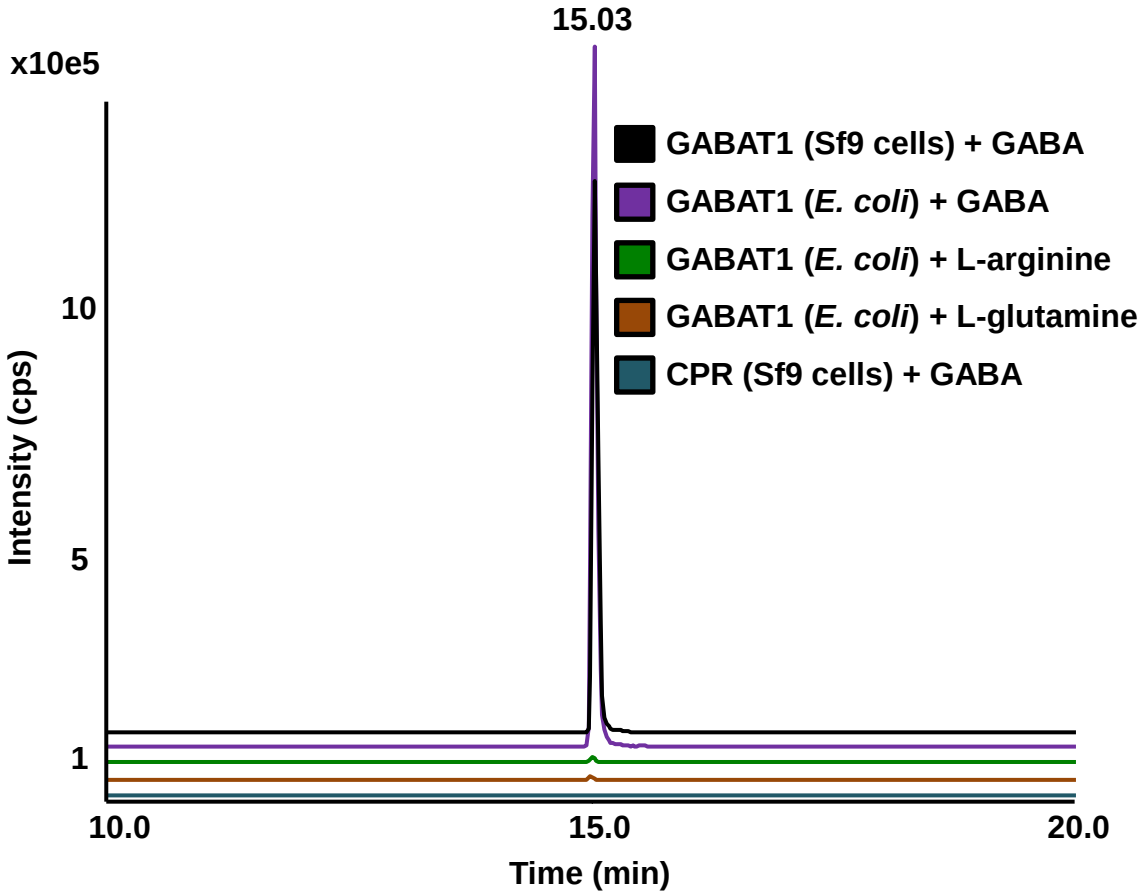


Figure S10. LC-MS/MS of enzyme assays with his-tag purified GABAT1. Recombinant GABAT1 was his-tag purified from *E.coli* PLUS E cells and used in enzyme assays with the substrate 22-hydroxycholesterol-26-al and GABA, L-arginine, or L-glutamine to determine the amino group donor. Sf9 cells infected with 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.

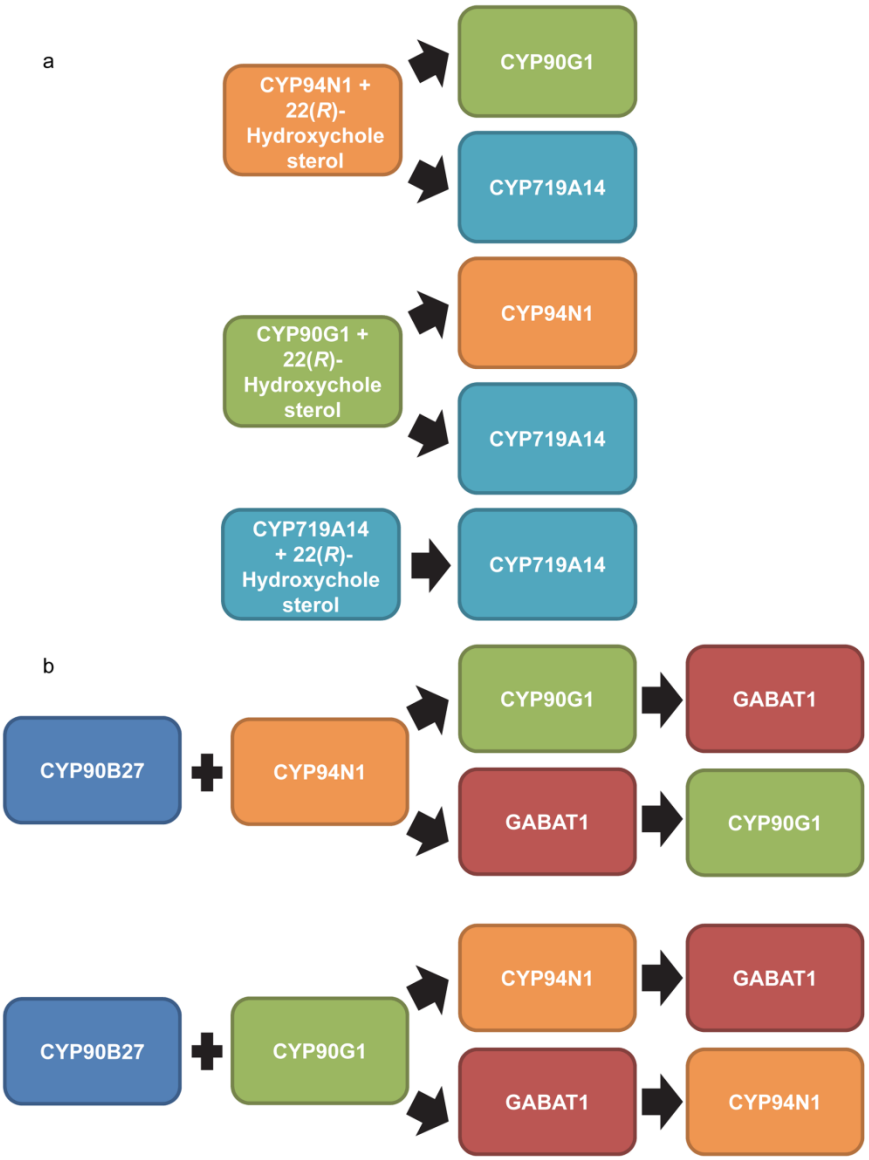


Figure S11. Enzyme assay workflow for clarification of cyclopamine biosynthetic pathway. Assays were performed with crude *S. frugiperda* Sf9 cells infected with baculovirus containing selected *Veratrum californicum* genes. Each arrow represents an extraction step; the resulting product was utilized 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 CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase), CYP90G1 (22-hydroxy-26-aminocholesterol 22-oxidase), and CYP719A14 (control cytochrome P450) each with pure 22(*R*)-hydroxycholesterol were incubated and extracted. Dried extracts from each were split and used as substrate in the reactions according to panel a. Final extracts were analyzed by GC-MS with results shown in Figure S12. (b) 8 assays of CYP90B27 (cholesterol 22-hydroxylase) and CYP94N1 and 8 assays of CYP90B27 and CYP90G1 were incubated and extracted. Dried extracts were split and used as substrate in 2 more enzyme assays, 4 reactions each as indicated by panel b. These were extracted then split into 2 more assays, 2 reactions each in reactions indicated by panel b. GABAT1 refers to 22-hydroxycholesterol-26-al transaminase. Products at each extraction step were analyzed by LC-MS/MS with results shown in Figure S7.

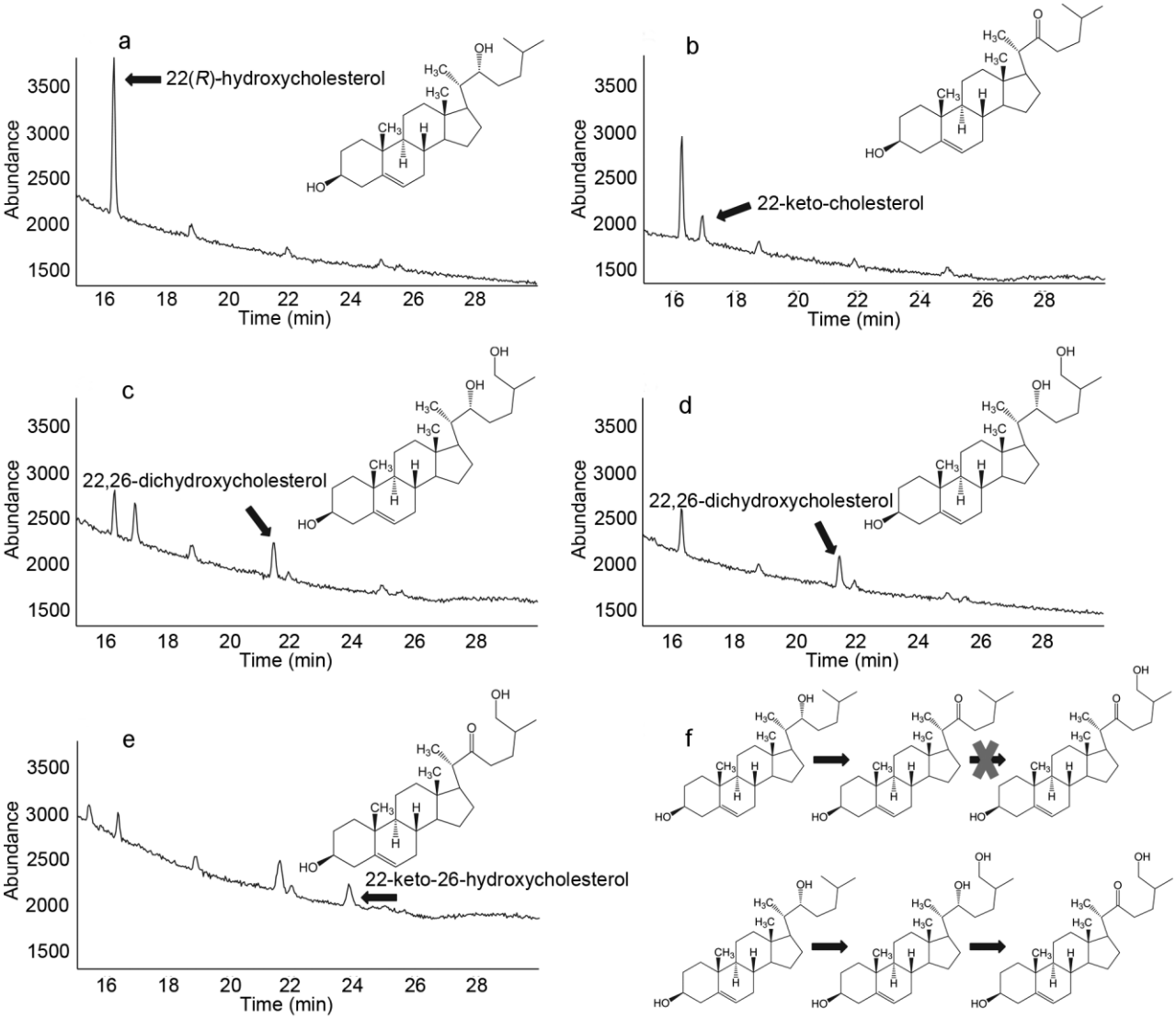


Figure S12. Enzyme assays for biosynthetic pathway order clarification in *Veratrum californicum* using GC-MS. Assays were completed according to Figure S11 a and analyzed by GC-MS in SIM mode. (a) Assay with an unrelated cytochrome P450 as control (CYP719A14, cheilanthifoline synthase from *A. mexicana)* 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 CYP90G1 (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 CYP90G1 + CPR and 22(*R*)-hydroxycholesterol was extracted and used as substrate in assay with CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase) + CPR. (d) Assay with CYP94N1 + CPR was extracted and used as substrate in assay with control cytochrome P450 + CPR. (e) Assay with CYP94N1 + CPR and 22(*R*)-hydroxycholesterol was extracted and used as substrate in assay with CYP90G1 + CPR. (f) Schematic of possible transformation order; X indicating which reaction was not observed. Ion 187 was extracted for the presented chromatograms.

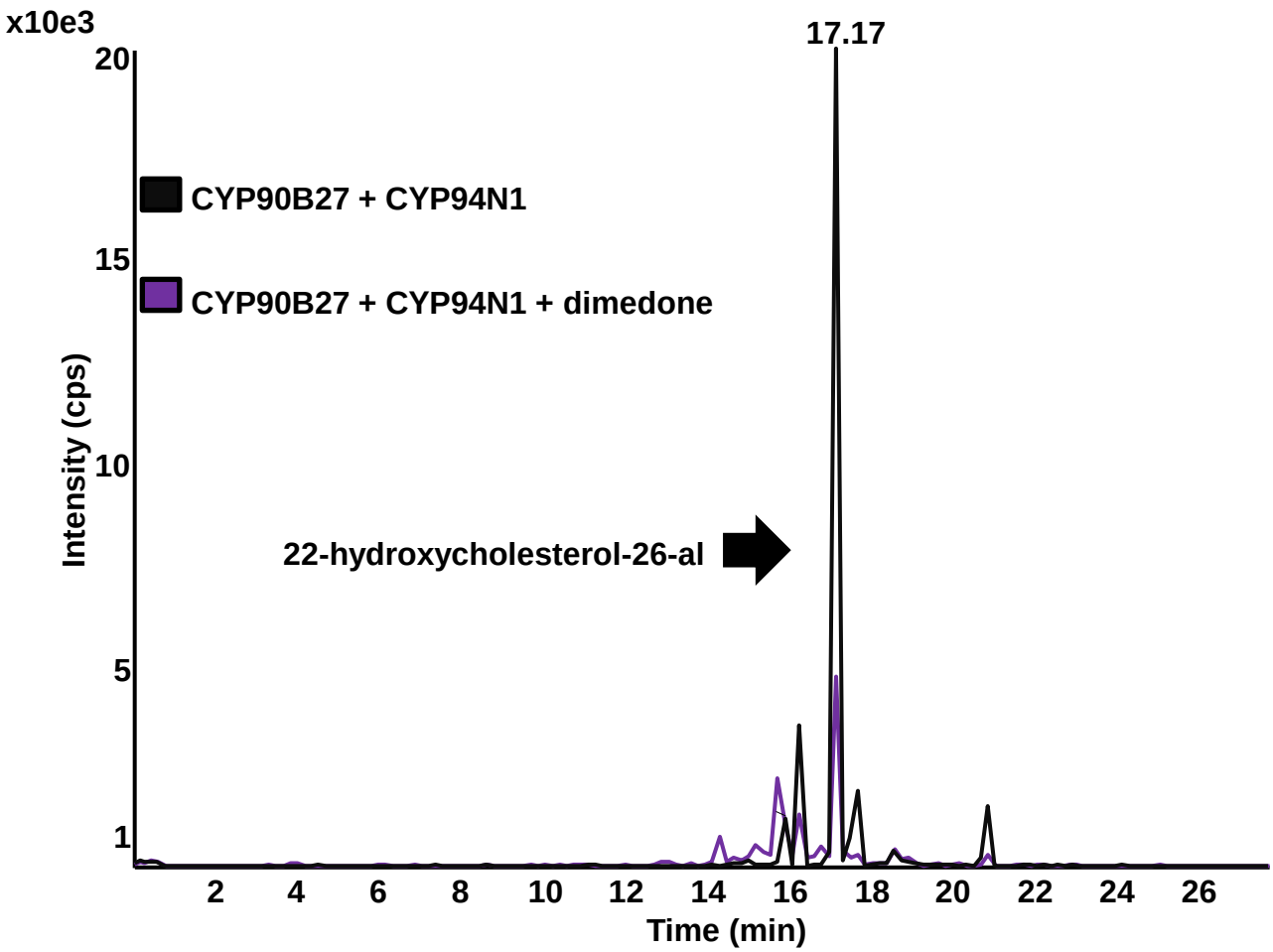


Figure S13. Trapping of enzymatically formed aldehyde intermediate. *S. frugiperda* Sf9 cell extracts expressing *Veratrum californicum* CYP90B27 (cholesterol 22-hydroxylase) co-expressed with *E. californica* cytochrome P450 reductase (CPR) and *V. californicum* CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase) co-expressed with CPR were mixed for enzyme assay using cholesterol provided by the crude cell extract as substrate. Assays performed without dimedone (black) and with dimedone (purple) were analyzed by LC-MS/MS. Chromatograms were obtained by overlay of Enhanced Product Ion scans (EPI) for molecular mass 417.

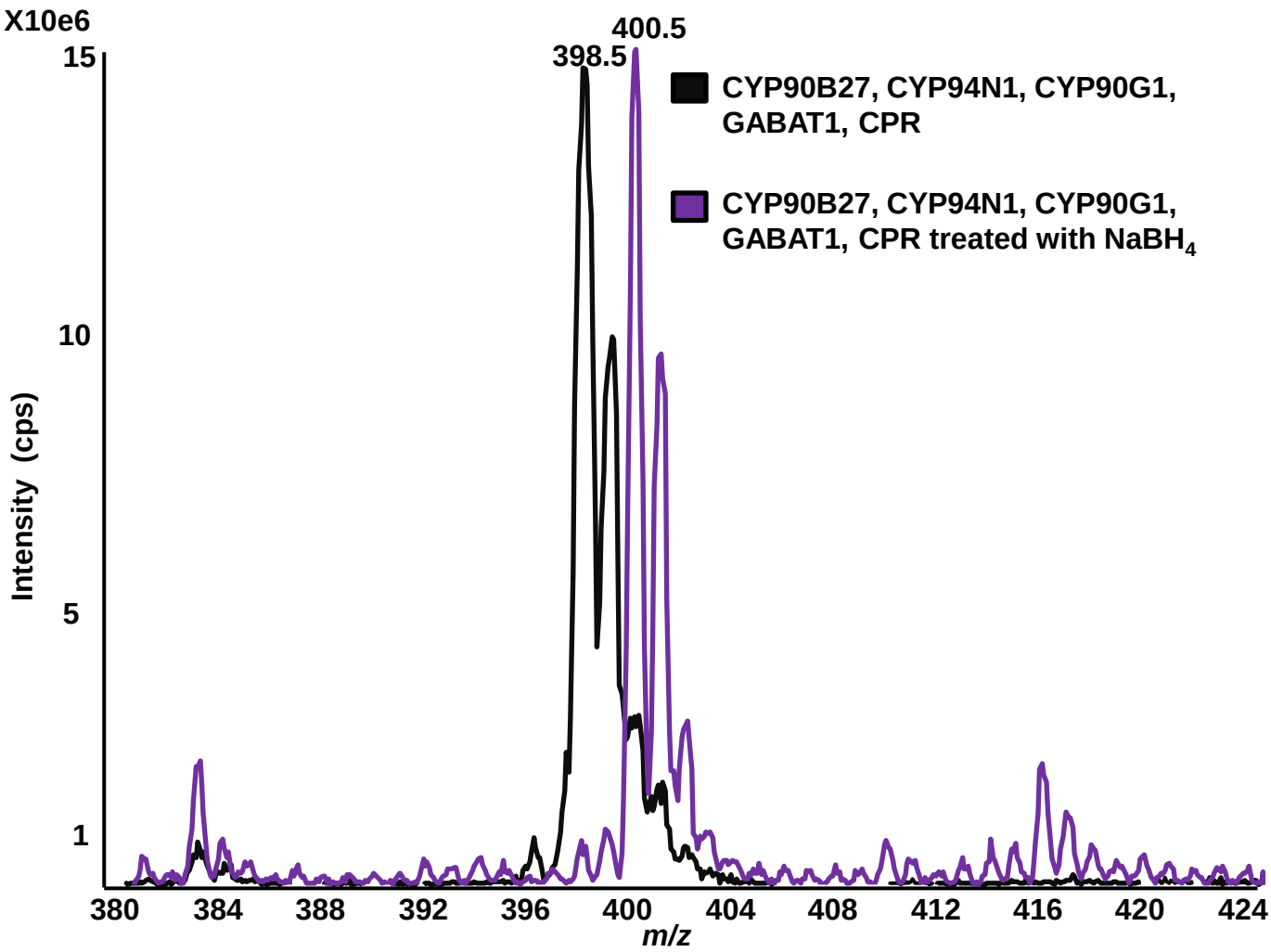


Figure S14. Derivatization of enzymatically formed verazine. *S. frugiperda* Sf9 cells expressing the *Veratrum californicum* genes CYP90B27 (cholesterol 22-hydroxylase), CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase), GABAT1 (22-hydroxycholesterol-26-al transaminase), CYP90G1 (22-hydroxy-26-aminocholesterol 22-oxidase), and *E. californica* cytochrome P450 reductase (CPR) were extracted and analyzed by LC-MS/MS either directly (black) or after treatment with $NaBH_4$ (purple). Enhance MS scans detecting ions 380-425 m/z are presented.

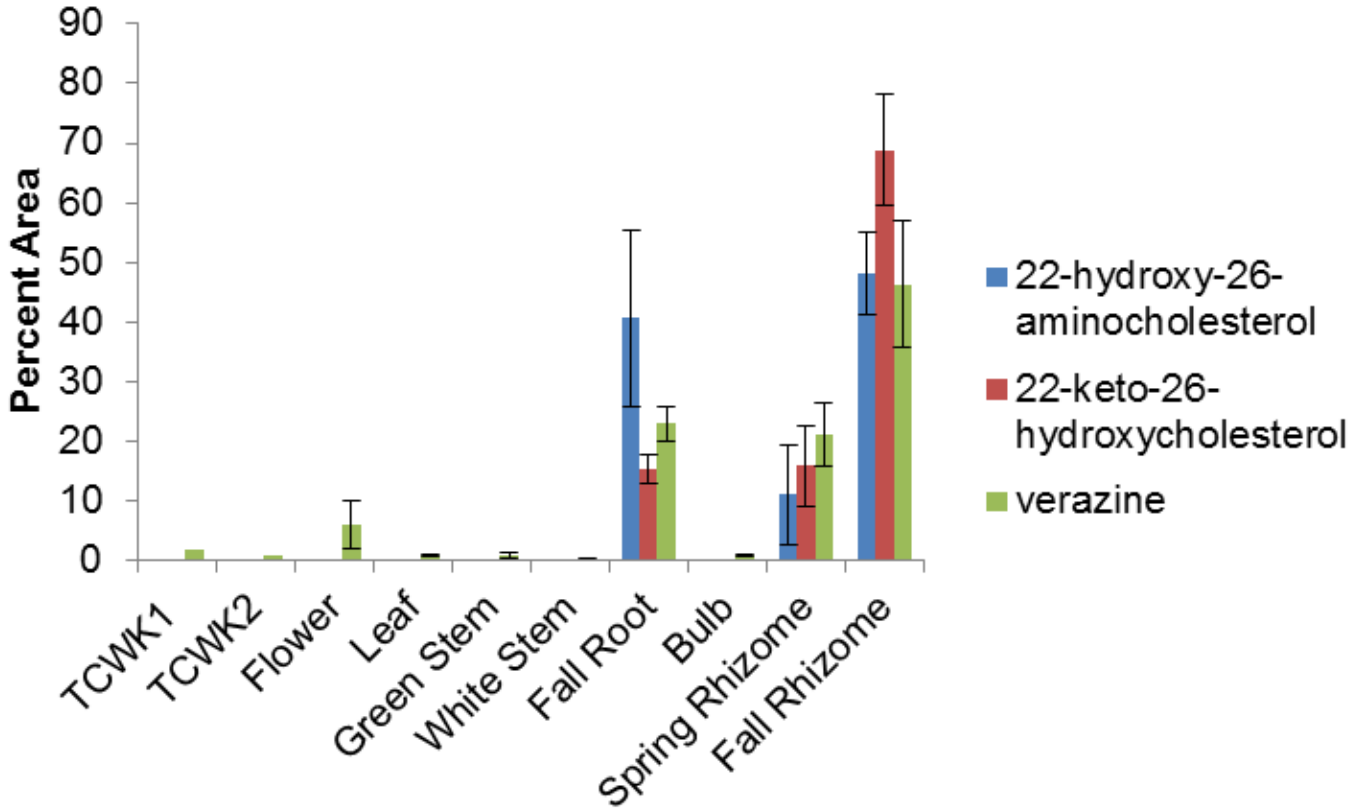
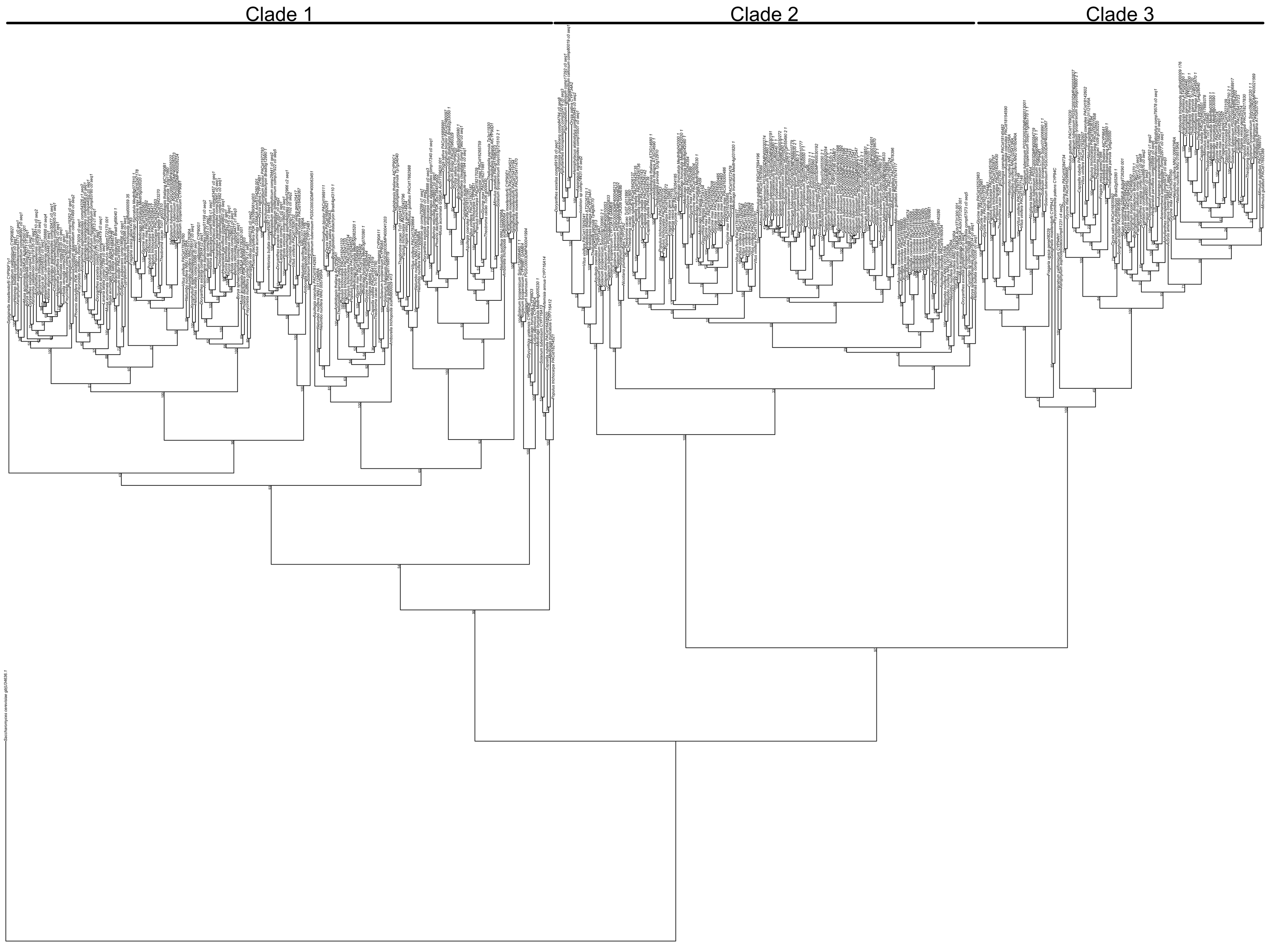


Figure S15. **Relative tissue specific accumulation of selected *Veratrum californicum* metabolites**. Relative quantities of metabolites 22-hydroxy-26-aminocholesterol, 22-keto-26-hydroxycholesterol, and verazine are shown for each tissue extract by percent of total peak area; error bars show standard deviation for three replicates as determined by LC-MS/MS. TCWK1 and TCWK2 stand for tissue culture one- and two weeks after transfer to new media, respectively.


Clade 1
Clade 2
Clade 3
Saccharomyces cerevisiae gb|U34636.1

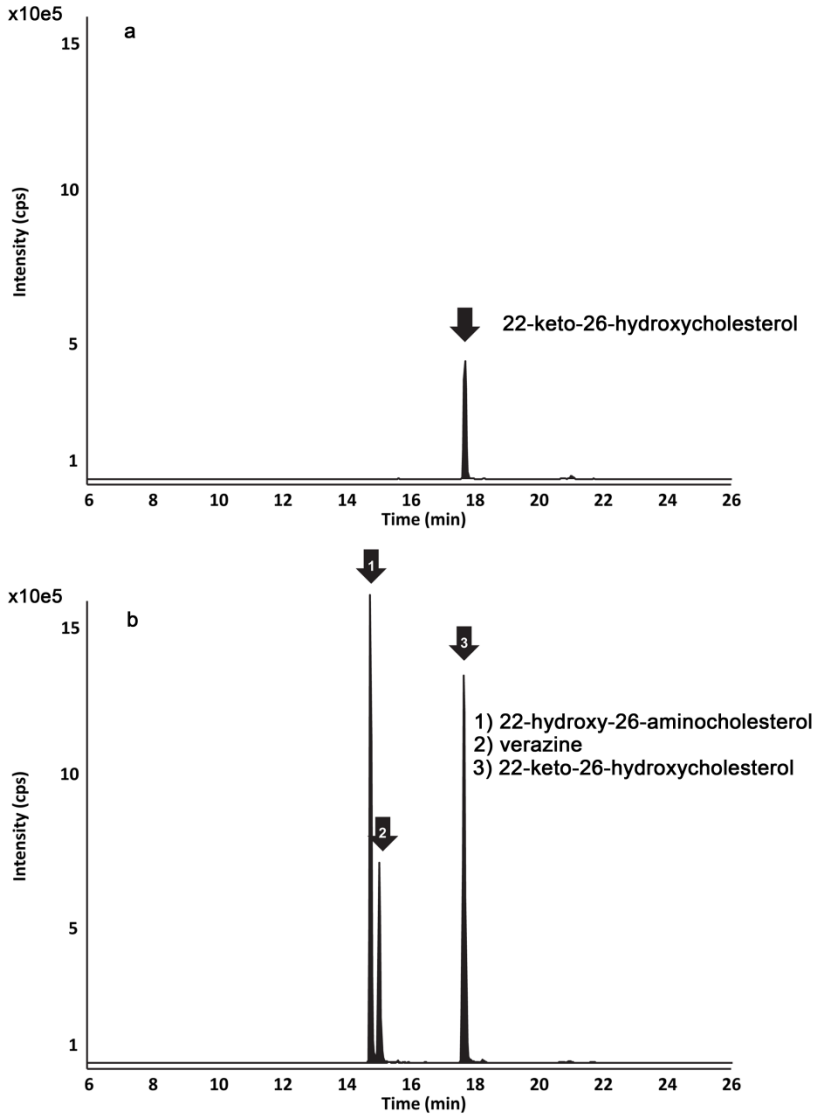


Figure S17. LC-MS/MS analysis of *Veratrum californicum* GABATs. *S. frugiperda* Sf9 cells we co-transformed with *E. californica* cytochrome P450 reductase (CPR), *V. californicum* cytochrome P450 enzymes: CYP90B27 (cholesterol 22-hydroxylase), CYP94N1 (22-hydroxycholesterol 26-hydroxylase/oxidase), CYP90G1 (22-hydroxy-26-aminocholesterol 22-oxidase), and (a) GABAT2 or (b) GABAT1 (22-hydroxycholesterol-26-al transaminase). Extracts were analyzed by LC-MS/MS; ions for each peak were combined and shaded for clarity.