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# Identification of *Vitis vinifera* (−)-α-terpineol synthase by in silico screening of full-length cDNA ESTs and functional characterization of recombinant terpene synthase

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**Abstract**

The flavour and aroma of certain *Vitis vinifera* grape varieties is dominated by volatile terpenes and small volatile aldehydes. Monoterpenes contribute to the final grape and wine aroma and flavour in form of free volatiles and as glycoside conjugates of monoterpene alcohols. Typical monoterpenol components of the cultivar Gewürztraminer and other aroma-rich grape varieties are linalool, geraniol, nerol, citronellol, and α-terpineol. In a functional genomics effort to identify genes for the formation of monoterpene alcohols in *V. vinifera*, a database of full-length cDNA sequences was screened in silico and yielded two clones for putative monoterpene synthases. The gene products were functionally characterized by expression in *Escherichia coli*, in vitro enzyme assay and gas chromatography-mass spectrometry (GC-MS) product identification as multi-product (−)-α-terpineol synthases.


*Keywords:* *Vitis vinifera*; Vitaceae; Grape vine; Wine flavour and aroma; Terpene cyclase

## 1. Introduction

Monoterpene natural products have a wide variety of functions in the interactions of plants with other organisms, including roles in direct defense against potential herbivores or pathogens, functions in indirect defense by olfactoric attraction of enemies of herbivores, as allelopathic compounds, in the attraction of pollinators by floral scents, or in the attraction of seed dispersing animals by the aromas or flavours of ripening fruits (Croteau et al., 2000). Because of their great appeal to humans as fruit aromas, flavours or floral fragrances, some monoterpenes have been selected for as important quality traits in traditional and modern plant breeding in agriculture, viticulture, and in horticulture. Certain monoterpenes also have beneficial functions as nutraceuticals. In particular, the possible anticancer protective properties of plant monoterpene alcohols, monoterpenols, such as perillyl alcohol, have received some considerable attention in recent years in cancer research and in food nutrition sciences (Burke et al., 2002; Crowell, 1999; Wagner et al., 2002). Although monoterpenols, together with other volatiles, such as aldehydes (e.g. hexenal) or ketones (e.g. pentanone), are important grape aroma and flavour components (Girard et al., 2002; Mateo and Jimenez, 2000), very little is known about the formation of monoterpenes in *V. vinifera*. A recent study by Luan and Wüst (2002) revealed that the exocarp and the mesocarp of grape berries contribute directly to monoterpene formation via the methyl-D-erythritol 4-phosphate (MEP) pathway. In earlier work, geranyl diphosphate synthase, a prenyl transferase for the formation of the immediate precursor of monoterpenes, was purified and characterized (Clastre et al., 1993). To our knowledge, no gene that is specific to the formation of monoterpenes has been identified and characterized in *V. vinifera*. Here we describe the identification and functional characterization of two *V. vinifera* cv. Gewürztraminer cDNAs that encode multi-product (−)-α-terpineol synthases.

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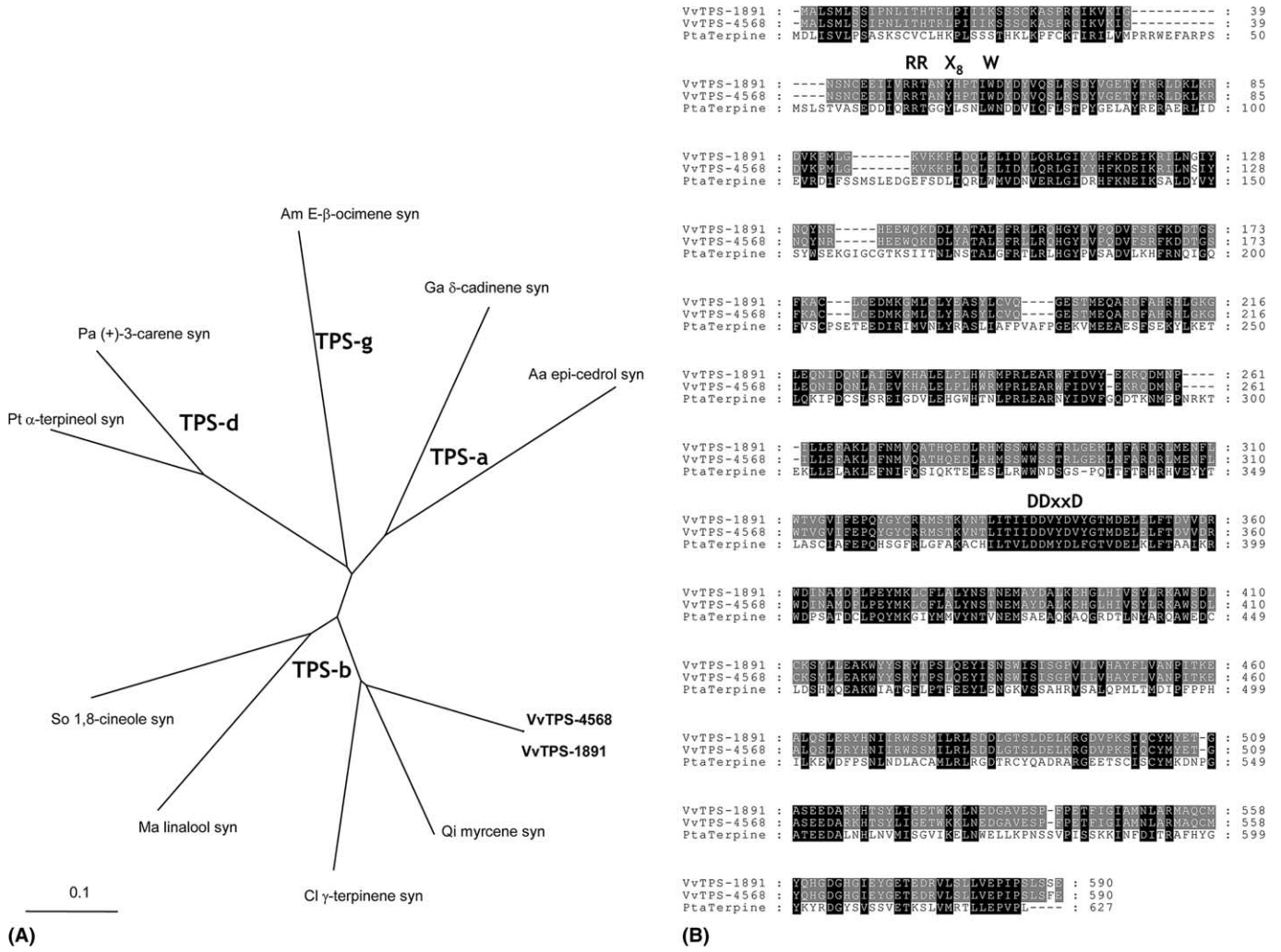


Fig. 1. Sequence relatedness of *V. vinifera* (−)-α-terpineol synthase VvTPS-1891 and Vv-TPS4568. (A) Radial unrooted phylogenetic tree of deduced amino acid sequences illustrating the relatedness of *V. vinifera* (−)-α-terpineol synthase VvTPS-1891 and VvTPS-4568 relative to known subfamilies of plant terpene synthases. VvTPS-1891 and VvTPS-4568 are located in the TPS-b subfamily with other angiosperm monoterpenes synthases. Am is *Antirrhinum majus*; Gs is *Gossypium arboreum*; Aa is *Artemisia annua*; Qi is *Quercus ilex*; Cl is *Citrus lemon*; Ma is *Mentha aquatica*; So is *Salvia officinalis*; Pt is *Pinus taeda*; Pa is *Picea abies*. (B) Amino acid alignment of VvTPS-1891, VvTPS-4568 and (−)-α-terpineol synthase from *Pinus taeda* showing only two amino acid differences between the two *V. vinifera* sequences yet these sequences are considerably divergent from the *P. taeda* sequence. The $RRX_8W$ and DDXXD motifs are highlighted. Shaded in black are amino acid positions identical in all three sequences. Shaded in grey are amino acid positions identical in two of three sequences.

## 2. Results and discussion

### 2.1. Identification in silico of grape full-length terpene synthase cDNAs

Although terpenoids are important attributes of grape and wine aromas and flavours, they are present in grapes only in small amounts when compared to the large quantities of monoterpenes accumulated in specialized anatomical structures of members of the *Lamiaceae*, *Rutacea*, *Pinaceae* or some other plant families. We therefore employed in silico screening of a large full-length cDNA EST database (http://www.vitigen.com) as an effective method for identification of potential grape monoterpene synthase genes. Plant genome and large EST databases have proven a very useful resource for the mining of genes of secondary metabolism including genes of terpenoid metabolism (Aubourg et al., 2002; Dudareva et al., 2003; Gang et al., 2001; Lange et al., 2000). Full-length cDNA sequence databases are a

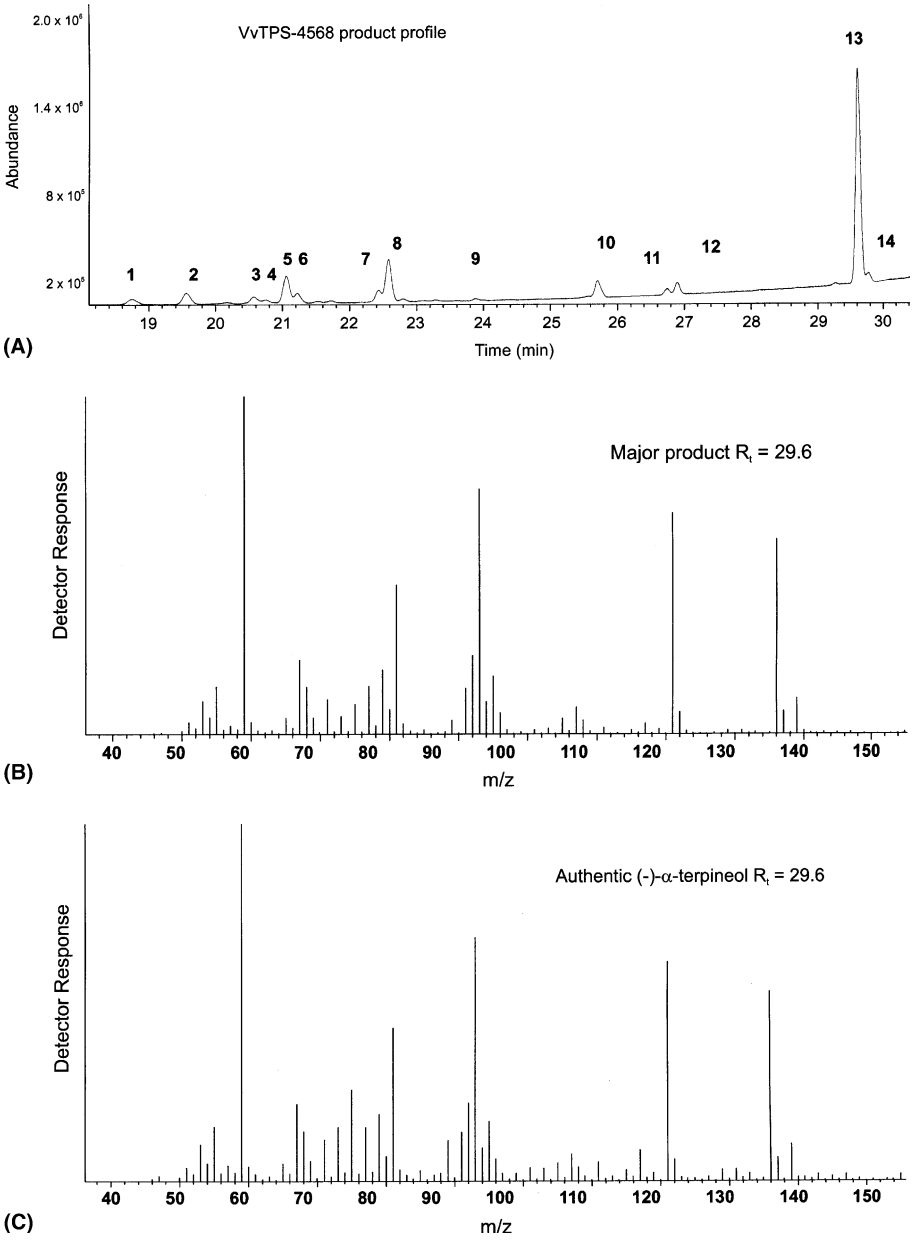


Fig. 2. Product identification of recombinant *V. vinifera* (−)-α-terpineol synthase Vv-TPS4568. GC-MS total ion chromatogram (A) of products produced by VvTPS4568 shows 14 monoterpenes including (−)-α-thujene (1), (−)-α-pinene (2), myrcene (3), sabinene (4), (+)-β-pinene (5), (−)-β-pinene (6), (−)-limonene (7), 1,8-cineol (8), terpinolene (9), (+)-trans-sabinene hydrate (10), (+)-cis-sabinene hydrate (11), (−)-cis-sabinene hydrate (12), (−)-α-tepineol (13), and (+)-α-terpineol (14). Mass spectra of the major product, peak 13, (B) are compared with the mass spectra of authentic (−)-α-terpineol synthase (C).

particularly powerful resource for functional genomics (Seki et al., 2002), because they allow for the identification of complete open reading frames (ORFs) as a means for improved gene annotation, and for functional expression of recombinant proteins for biochemical characterization. As a grape variety, Gewürztraminer was chosen because of its monoterpene-rich aroma profile. The full-length cDNA database used in our data search contained entries from an array of grape organs and tissues, including leaves, stems, tendrils, and developing berries (Driesel et al., 2003). By screening in silico of the sequence database using known monoterpene synthase nucleotide and amino sequences of angiosperms (Aubourg et al., 2002; Bohlmann et al., 1998), we identified two monoterpene synthase-like genes. The cDNA clones VitiC1891 (*VvTPS-1891*) and VitiC4568 (*VvTPS-4568*) were identical in their longest open reading frames except for two amino acid residues and resembled known monoterpene synthases of the *TPS-b* group (Aubourg et al., 2002; Bohlmann et al., 1998) (Fig. 1). The longest ORF of both clones encode for a deduced protein sequence of 590 amino acids, and a predicted isoelectric point of 5.86. The deduced amino acid sequences are most closely related to myrcene synthase from *Quercus ilex* (CAC41012) among the known plant monoterpene synthase sequences (Fischbach et al., 2000) and only distantly related to (−)-α-terpineol synthase (AAO61227) from *Pinus taeda* (Phillips et al., 2003) (Fig. 1). The sequences contain the conserved features of plant monoterpene synthases including the active side DDxxD motif (Starks et al., 1997; Whittington et al., 2002) and the $RRx_8W$ motif (Bohlmann et al., 1998) that is involved in isomerization of geranyl dihosphate (GPP) in the cyclization reaction of limonene synthase from *Mentha spicata* (Williams et al., 1998). The amino-terminal extension of 48 amino acids upstream of the $RRx_8W$-motif and sequence analysis using ChloroP 1.1-software (http://www.cbs.dtu.dk/services/ChloroP/) predicted that both cDNA clones encode preproteins containing an N-terminal transit peptide for plastid import of approximately 28–48 amino acids.

### 2.2. Functional identification of (−)-α-terpineol synthase cDNAs

The coding sequences of cDNA *VvTPS-1891* and *VvTPS-4568*, truncated at the $RRx_8W$ site, were subcloned into the expression vector pET100/D-TOPO® and expressed by IPTG-induction in *E. coli* strain BL21 codon plus. Recombinant, expressed proteins were tested for terpene synthase activity using GPP (a 10-carbon prenyl diphosphate), farnesyl diphosphate (FPP, 15-carbon), and geranylgeranyl diphosphate (GGPP, 20-carbon) as substrates in separate in vitro enzyme assays. GPP is the common substrate for all plant monoterpene synthases described to date, FPP is the substrate for sesquiterpene synthases, and GGPP is the substrate for diterpene synthases (Cane, 1999; MacMillan and Beale, 1999; Wise and Croteau, 1999). In our assays, product formation was detected with GPP, but not with FPP or GGPP. Products derived from GPP were identified by GC-MS after organic extraction of the aqueous assay mixture. The GC trace of VvTPS-4568 enzyme product showed 14 peaks corresponding to compounds 1–14, including the major peaks (−)-α-terpineol (**13**) (50.1%), 1,8-cineol (**8**) (11.8%), and (+)-β-pinene (**5**) (8.5%) and the minor peaks (−)-α-thujene (**1**), (−)-α-pinene (**2**), myrcene (**3**), sabinene (**4**), (−)-β-pinene (**6**), (−)-limonene (**7**), terpinolene (**9**), (−)-*trans*-sabinene hydrate (**10**), (+)-*cis*-sabinene hydrate (**11**), (−)-*cis*-sabinene hydrate (**12**), and (+)-α-terpineol (**14**) (Fig. 2, Table 1). Control assays with extracts of *E. coli* (same strain) lacking a cDNA clone did not yield monoterpene products. cDNA clone *VvTPS-1891* produced an identical product profile as *VvTPS-4568*. Therefore, both *V. vinifera* genes were designated as (−)-α-terpineol synthases. Although the formation of multiple products is a common feature of plant monoterpene synthase, a monoterpene that produces predominantly (−)-α-terpineol synthase has not been previously described from an angiosperm and the exact product profiles and sequences are substantially different from the gymnosperm (−)-α-terpineol synthase from loblolly pine which is a member of the *TPS-d* subfamily (Phillips et al., 2003).

Table 1
Product profile of *V. vinifera* (−)-α-terpineol synthase VvTPS-4568 showing six oxygenated monoterpenes and eight monoterpene olefins

| Compound | *Rt* | Total (%) |
|---|---|---|
| (−)-α-thujene (1) | 18.7 | 2.8 |
| (−)-α-pinene (2) | 19.6 | 4.3 |
| Myrcene (3) | 20.6 | 2.5 |
| Sabinene (4) | 20.8 | 1.3 |
| (+)-β-pinene (5) | 21.1 | 8.5 |
| (−)-β-pinene (6) | 21.2 | 3.0 |
| (−)-limonene (7) | 22.4 | 2.8 |
| 1,8-cineol (8) | 22.6 | 11.8 |
| Terpinolene (9) | 23.9 | 0.7 |
| (−)-*trans*-sabinene hydrate (10) | 25.7 | 5.6 |
| (+)-*cis*-sabiene hydrate (11) | 26.7 | 1.7 |
| (−)-*cis*-sabinene hydrate (12) | 26.9 | 3.2 |
| (−)-α-terpineol (13) | 29.6 | 50.1 |
| (+)-α-terpineol (14) | 29.8 | 1.9 |

Numbers in parentheses indicate peaks in Fig. 2.

## 3. Conclusions

The cDNAs *VvTPS-1891* and *VvTPS-4568* present the first monoterpene synthases cloned and characterized from *V. vinifera*, a species that is appreciated for a nat-

ural product aroma and flavour bouquet that is composed of a suite of monoterpenes, mainly monoterpene alcohols, and other volatiles. The major and minor products of grape vine multifunctional (−)-α-terpineol synthase are known components of aroma-rich grape cultivars such as Gewürztraminer or Muscat (Girard et al., 2002; Karagiannis et al., 2000). While it is possible that α-terpineol also arises during wine production from non-enzymatic rearrangements of acyclic monoterpenes such as geraniol, nerol, and linalool, as is known from acid treatment of grape juice (Girard et al., 2002), our results of cloning and functional identification of α-terpineol synthase provide evidence that *V. vinifera* has the genetic and biochemical capacity to form this monoterpenol compound directly from GPP by mechanism of a typical monoterpene synthase. The identification of grape monoterpene synthase cDNAs will facilitate the cloning of additional genes of the *TPS* family in *V. vinifera* and will facilitate future molecular and physiological studies of the regulation of flavour and aroma formation during fruit development and ripening. Grape *TPS* genes will provide useful molecular tools to monitor the impact of viticulture practices and environmental factors on grape and wine aroma quality. This and potentially other *TPS* genes from *V. vinifera* may be developed into molecular markers to aid in breeding and improvement of varieties with superior aroma, flavor and nutraceutical quality traits.

## 4. Experimental

### 4.1. Screening of Vitis vinifera full-length cDNAs

The *Vitis vinifera* L., cv. Gewürztraminer full-length cDNA EST database (http://www.vitigen.com) of VitiGen AG (Siebeldingen, Germany) was screened using standard BLAST algorithms (Altschul et al., 1990) and known monoterpene synthase sequences (Aubourg et al., 2002). Two full-length cDNA clones, VitiC1891 (*VvTPS-1891*) and VitiC4568 (*VvTPS-4568*) were retrieved from the VitiGen AG plasmid stock collection.

### 4.2. Subcloning and expression of cDNA in E. coli

ORFs of *VvTPS-1891* and *VvTPS-4568* were amplified by PCR using Pfu-Turbo (Stratagene, La Jolla, CA) with the forward primer MTPet5R (CAC CAT GAG GCG AAC TGC AAA CTA CCA T) for amplicons truncated at the $RRx_8W$ site and subcloned according to established procedures (Fäldt et al., 2003). Reverse primers were either MTPet1891stp (TTA TTC AGA ACT CAA ACT GGG AA) for *VvTPS-1891* or MTPet34569stp (TTA TTC AAA ACT CAA ACT GGG A) for *VvTPS-4568*. PCR product were then directly cloned into the pET100/D-TOPO directional expression vector (Invitrogen, Carlsbad, CA). The recombinant expression plasmids were rescued and maintained in *E. coli* TOP10 F' cells, analyzed by PCR using insert and vector based primers, and sequenced to verify cloning. Plasmids were transformed into *E. coli* BL21-CodonPlus (Stratagene) cells for expression of terpene synthase genes.

### 4.3. Terpene synthase enzyme assays and GC-MS analysis

To functionally express terpene synthases, *E. coli* BL21-CodonPlus(DE3)/pET100/D-*VvTPS-1891* and *E. coli* BL21-CodonPlus(DE3)/pET100/D-*VvTPS-4568* were grown in 5 ml LB cultures with ampicillin and induced with 200 μM IPTG, centrifuged and resuspended in assay butter (30 mM HEPES, pH 7.2, 7.5 mM $MgCl_2$, 20 μM $MnCl_2$, 5% glycerol, 5 mM DTT), and sonicated as described (Fäldt et al., 2003). Cell lysates were cleared by centrifugation and the supernatant was removed and assayed for terpene synthase activity with 55 μM GPP (Echelon Bioscience Inc., Salt Lake City, UT) as described (Fäldt et al., 2003). A 1-ml pentane overlay was used to trap terpene products during the assays and in all cases, after incubation at 30 °C for 1 h, the reaction mixture was further extracted with pentane ($3 \times 1$ ml). The combined extract was processed with water to remove small organic acids according to Peters et al. (2000). Washed pentane extracts were evaporated to 50–100 μl and 1 or 2 μl were injected and analyzed by GC-MS as detailed in Martin et al. (2002) and Fäldt et al. (2003). Extracts of *E. coli* BL21-CodonPlus (DE3) transformed with either plasmid without the insert were assayed as above to control for product formation independent of any *V. vinifera* cDNA clone. Assays using FPP and GGPP were as described in Martin et al. (2002). GC-MS analysis was performed on an Agilent 6890 Series GC System coupled to an Agilent 5973 Network Mass Selective Detector (70 eV) using two columns of different polarities. For all analyses, the injector port was maintained at 220 °C, the column flow was held constant at 0.7 ml/min, the MS operated at 70 eV and all capillary columns were of the dimensions 0.25 mm i.d. × 30 m with 0.25-μm film. To analyze monoterpenes using a DB-WAX capillary column (J&W Scientific, Palo Alto, CA, USA) the program was as follows: an initial temperature of 40 °C (4 min hold) was increased to 150 °C at 4 °C $min^{-1}$ followed by a 20 °C $min^{-1}$ ramp until 230 °C (5 min hold). The temperature program for the HP-5 capillary column (Agilent Technologies, Palo Alto, CA, USA) began at 40 °C (2 min hold) with an initial ramp of 3 °C $min^{-1}$ until 180 °C followed by a 20 °C $min^{-1}$ until 300 °C (3 min hold). Analysis of chiral compounds was achieved using a Cyclosil B column (Agilent Technologies, Palo Alto, CA, USA) capillary column. The oven was programmed from 55 °C (2 min hold) to 230 at

5 °C/min. All other parameters were as mentioned above. All products were identified using authentic standards and/or Wiley library matches.

### 4.4. Sequence analysis

Deduced amino acid sequences and predicted pI's were generated using the entire open reading frame within EditSeq 5.00 (DNASTAR Inc.). Amino acid alignments were made with ClustalX (http://www-igbmc.u-strasbg.fr/BioInfo/) and visualized with GeneDoc (http://www.psc.edu/biomed/genedoc/). Phylogenetic trees were generated using the nearest neighbor joining method though ClustalX and visualized with TreeView (http://taxonomy.zoology.gla.ac.uk/rod/treeview.html).

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