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# A chicory cytochrome P450 mono-oxygenase CYP71AV8 for the oxidation of (+)-valencene

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ABSTRACT

**Chicory (*Cichorium intybus* L.), which is known to have a variety of terpene-hydroxylating activities, was screened for a P450 mono-oxygenase to convert (+)-valencene to (+)-nootkatone. A novel P450 cDNA was identified in a chicory root EST library. Co-expression of the enzyme with a valencene synthase in yeast, led to formation of *trans*-nootkatol, *cis*-nootkatol and (+)-nootkatone. The novel enzyme was also found to catalyse a three step conversion of germacrene A to germacra-1(10),4,11(13)-trien-12-oic acid, indicating its involvement in chicory sesquiterpene lactone biosynthesis. Likewise, amorpha-4,11-diene was converted to artemisinic acid. Surprisingly, the chicory P450 has a different regio-specificity on (+)-valencene compared to germacrene A and amorpha-4,11-diene.**


## 1. Introduction

Many sesquiterpenes are of significant importance for the flavour and fragrance industry. Hydroxylation of these terpenes often adds to their sensory properties, and is therefore an essential step in the production of flavour molecules. The regio- and stereoselective introduction of a hydroxyl group into an unactivated organic compound by means of organic synthesis is still challenging [1]. For this reason, hydroxylating enzymes such as cytochrome P450 mono-oxygenases are considered as an alternative way to introduce regioselective modifications [2] and have great potential for application in the production of natural flavour molecules.

(+)-Nootkatone is an important oxidised sesquiterpene for the flavour and fragrance industry. It has a characteristic grapefruit-like flavour and a low odour threshold [3]. Natural (+)-nootkatone can be extracted from grapefruit [4]. Since grapefruit material is limited on the world market, synthetic (+)-nootkatone produced from (+)-valencene is predominantly used in commercial applications, also for the flavour market [5]. Chemical oxidation of valencene requires the use of *tert*-butyl chromate [6], which is a carcinogenic substance. Alternatively non-carcinogenic *tert*-butyl peracetate [7] or *tert*-butyl hydroperoxide can be used which are highly flammable and corrosive compounds.

(+)-Nootkatone may also be produced from (+)-valencene via biotechnological approaches in recombinant organisms using the enzymes involved in its biosynthesis [5]. The biosynthetic route to (+)-nootkatone in grapefruit has not been established experimentally, but has been suggested to start from valencene on which a regioselective allylic hydroxylation results in formation of 2-hydroxyvalencene (or nootkatol), followed by oxidation to (+)-nootkatone [4,5].

Several enzymes have been reported to mediate oxidation of (+)-valencene to (+)-nootkatone or to its precursor nootkatol. Engineered cytochrome P450s from *Pseudomonas putida* and *Bacillus megaterium* were shown to convert valencene into nootkatone [8,9]. Recently, a (+)-valencene dioxygenase from *Pleurotus sapidus* was demonstrated to have this activity. For plants, several cytochrome P450 enzymes capable of oxidising valencene have been described. The premnaspirodiene oxygenase from *Hyoscyamus*

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*muticus* [10] was shown to catalyse mono-oxygenation of (+)-valencene to nootkatol. Mutations in substrate recognition regions of this enzyme further improved the catalytic efficiency of nootkatol formation [10]. De Kraker et al. describe oxidation of a number of sesquiterpenes by a microsomal extract from taproot of *Cichorium intybus* (chicory), in which P450 enzymes are represented. Among the sesquiterpene olefins that were tested as substrate were germacrene A, which is naturally present in chicory as precursor for the synthesis of bitter sesquiterpene lactones, but also sesquiterpenes that do not occur in chicory root, such as amorpha-4,11-diene and (+)-valencene. In the case of (+)-valencene, both *trans*-nootkatol and (+)-nootkatone were found as reaction products [11]. Plant microsomal extracts, such as the chicory root extract, likely contain a variety of P450 enzymes. The formation of bitter sesquiterpene lactones in root tissue requires several oxygenation steps, at different regions of the sesquiterpene carbon scaffold. Recently, a cytochrome P450 from chicory has been cloned which catalyzes oxidation of germacrene A and amorpha-4,11-diene at the allylic position C12 [12]. Our aim was to clone a chicory cytochrome P450 which is capable of catalyzing the regioselective oxidation of (+)-valencene and which can be used for the biotechnological production of (+)-nootkatone.

## 2. Materials and methods

### 2.1. Isolation and cloning of P450 gene from chicory

An expressed sequence tag (EST) database of a cDNA library derived from the taproot of chicory was produced using the Roche 454 sequencing platform. Sequences were assembled using an *in-house* bioinformatics facility and candidate P450s contigs were identified by sequence homology to known sesquiterpene hydroxylases. RACE PCR (Clontech) was used to obtain the sequence of the 5′-region of the candidate contig 28771.

The full length gene was amplified from chicory cDNA using high fidelity Phusion polymerase (Finnzymes) with the addition of NotI and PacI restriction sites and was subsequently cloned to pGEM-T Easy (Promega). Three individual clones were fully sequenced showing no sequence variation. The deduced protein sequence was aligned with germacrene A oxidase (acc. no. GU256644) [12], premnaspirodiene oxygenase (acc. no. EF569601) [10] and amorpha-4,11-diene oxidase (acc. no. DQ315671) [13,14] using Clustal W [15]. The sequence of the gene was deposited in the NCBI GenBank Nucleotide database under accession number HQ166835. The sequence was also submitted to David Nelson's cytochrome P450 homepage (http://drnelson.uthsc.edu/cytochromeP450.html) and was assigned the name CYP71AV8 [16].

### 2.2. Co-expression of CYP71AV8 with terpene synthases in yeast

The full length gene was re-cloned, using the *Not*I/*Pac*I restriction sites, into the yeast expression vector pYEDP60 [17] which was modified to contain *Pac*I and *Not*I sites at the polylinker. The candidate chicory P450 was co-transformed with either a germacrene A synthase [18], amorpha-4,11-diene synthase [14] or a valencene synthase [19] into the into yeast strain WAT 11 expressing *Arabidopsis* ATR1 NADPH-cytochrome P450 reductase [20]. Germacrene A synthase was cloned into pYEDP80 vector [17] with TRP1 auxotrophic selection marker using *Bam*HI and *Eco*RI restriction sites. Amorpha-4,11-diene synthase and valencene synthase were both cloned into pYES3/CT yeast expression vector (Invitrogen) with TRP1 selection marker using *Bam*HI and *Not*I restriction sites. No terminal tags were added in these constructs. After transformation yeast clones containing the chicory P450 and terpene synthases were selected on Synthetic Dextrose minimal medium (0.67% Difco yeast nitrogen base medium without amino acids, 2% D-glucose, 2% agar) supplemented with amino acids, but omitting uracil, adenine sulphate and L-tryptophane for auxotrophic selection of transformants.

A starter yeast culture was grown overnight at 30 °C in 5 ml of Synthetic Galactose minimal medium (0.67% Difco yeast nitrogen base medium without amino acids, 2% D-galactose, amino acids, but omitting uracil, adenine sulphate and L-tryptophane), The starter culture was diluted to $OD_{600}$ of 0.05 in 50 ml of Synthetic Galactose minimal medium and incubated at 200 rpm at 30 °C. The culture was overlaid with 5 ml of *n*-dodecane [21] when the $OD_{600}$ was in the range from 0.8 to 1 and cultivation was continued for 3 days. The *n*-dodecane layer was collected and centrifuged at 1200 rpm for 10 min, diluted threefold in ethyl acetate, dried using anhydrous $Na_2SO_4$ and then used for GC–MS analysis. In co-expression experiments with amorpha-4,11-diene synthase and germacrene A synthase 7 ml of the yeast culture was further extracted three times with 2 ml ethyl acetate, concentrated, dehydrated using anhydrous $Na_2SO_4$ and used for GC-MS analysis. For analysis of artemisinic acid and germacra-1(10),4,11(13)-trien-12-oic acid, samples were first methylated using diazomethane.

### 2.3. GC-MS analysis

Analytes from 1 μL samples were separated using a gas chromatograph (5890 series II, Hewlett-Packard) equipped with a 30 m × 0.25 mm, 0.25 mm film thickness column (ZB-5, Phenomenex) using helium as carrier gas at flow rate of 1 ml/min. The injector was used in splitless mode with the inlet temperature set to 250 °C. The initial oven temperature of 45 °C was increased after 1 min to 300 °C at a rate of 10 °C/min and held for 5 min at 300 °C. The GC was coupled to a mass-selective detector (model 5972A, Hewlett-Packard). Compounds were identified by comparison of mass spectra and retention times (rt) with those of the following authentic standards: germacrene A, germacra-1(10),4,11(13)-trien-12-ol, germacra-1(10),4,11 (13)-trien-12-al [11], amorpha-4,11-diene, artemisinic alcohol, dihydroartemisinic alcohol, artemisinic aldehyde, artemisinic acid [22], (+)-valencene, *trans*-nootkatol, *cis*-nootkatol (Isobionics) and (+)-nootkatone (Fluka). Quantification of sesquiterpenes was conducted by determination of total ion count (TIC) peak area of the sesquiterpene peaks from three independent fermentation experiments. Absolute concentration of sesquiterpenes was calculated from the peak area by comparison to a standard curve prepared by measuring a dilution series of authentic standards with known concentrations.

## 3. Results

### 3.1. Isolation of a novel P450 gene from chicory

A cDNA library was created from root tissue of *C. intybus*. This library was analyzed by sequencing using the Roche 454 sequencing platform, which resulted in 575 945 EST sequences of on average 400 bp in size. By sequence assembly, 40 847 contigs were formed from which 31 787 singletons remained.

The sequences were combined with 35 973 *C. intybus* EST sequences from GenBank, and interrogated for sequences with homology to the cytochrome P450 sequences of premnaspirodiene oxygenase from *H. muticus* [10] and amorpha-4,11-diene oxidase from *Artemisia annua* [13,14]. Two sequences with high similarity to both genes were identified, one of which encoded the recently described *C. intybus* germacrene A oxidase [12]. The second sequence encodes a novel protein CYP71AV8 with 50% sequence identity to premnaspirodiene oxygenase, 78% to amorpha-4,11-diene oxidase and 81% to the germacrene A oxidase from *C. intybus*.

**Table 1**
Quantification of valencene-derived products in the *n*-dodecane layer after yeast fermentation.

| Enzymes expressed | Products | Quantity mg/l yeast culture (mean ± S.D., *n* = 3) |
|---|---|---|
| VS | (+)-valencene | 1.36 ± 0.05 |
| VS + CYP71AV8 | (+)-valencene | 0.15 ± 0.02 |
| | *trans*-nootkatol | 0.92 ± 0.07 |
| | *cis*-nootkatol | 0.11 ± 0.03 |
| | (+)-nootkatone | 0.04 ± 0.00 |

VS = valencene synthase.

### 3.2. CYP71AV8 is a (+)-valencene oxidase and produces (+)-nootkatone in vivo

To assess its value in a biotechnological production platform, the activity of CYP71AV8 on (+)-valencene was tested by co-expression of the enzyme with a valencene synthase in yeast. In yeast cultures co-expressing valencene synthase and CYP71AV8 the majority of (+)-valencene had disappeared compared to control cultures without P450 (Table 1). As novel product, the predominant peak was identified as *trans*-nootkatol (rt = 16.53 min). A smaller peak of *cis*-nootkatol was detected (rt = 16.36 min) (Fig. 1). At a retention time of 17.51 min a small peak of (+)-nootkatone was detected. A

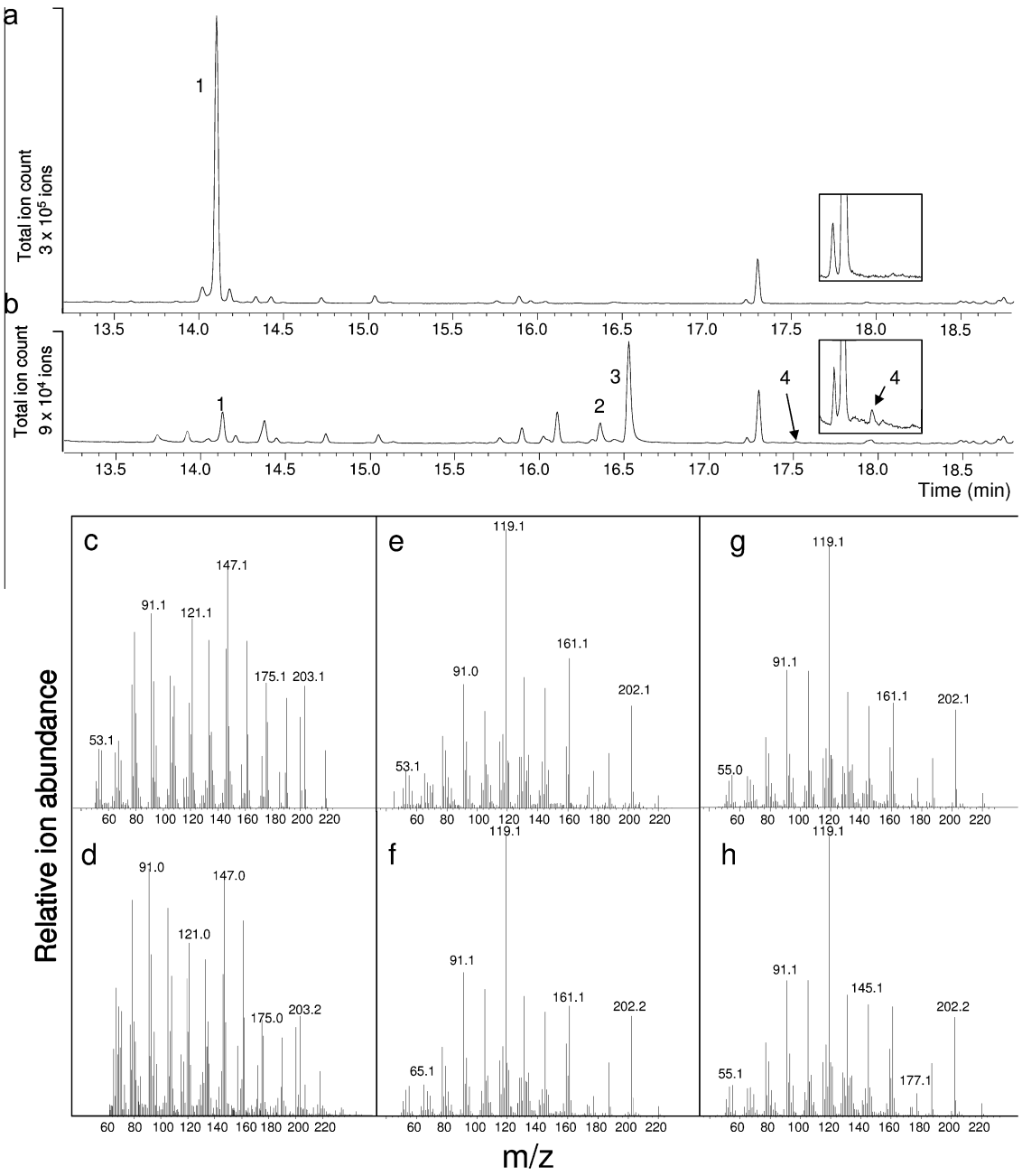


**Fig. 1.** GC-MS analysis of in vivo hydroxylation of (+)-valencene by CYP71AV8 to nootkatol and (+)-nootkatone. Chromatogram of the *n*-dodecane layer from (a) yeast strain WAT11 producing (+)-valencene (1) is compared to (b) the yeast strain WAT11 expressing valencene synthase and CYP71AV8 where (+)-valencene (1) is transformed into *cis*-nootkatol (2), *trans*-nootkatol (3) and trace amounts of (+)-nootkatone (4). Inserts in the chromatograms zoom in on the region of 17.2 to 17.7 min of the chromatograms. The *y*-axis scales of the chromatograms are identical. Mass spectra for (+)-nootkatone (c), *cis*-nootkatol (e) and *trans*-nootkatol (g) produced by yeast and (+)-nootkatone (d), *cis*-nootkatol (f) and *trans*-nootkatol (h) standards are shown.

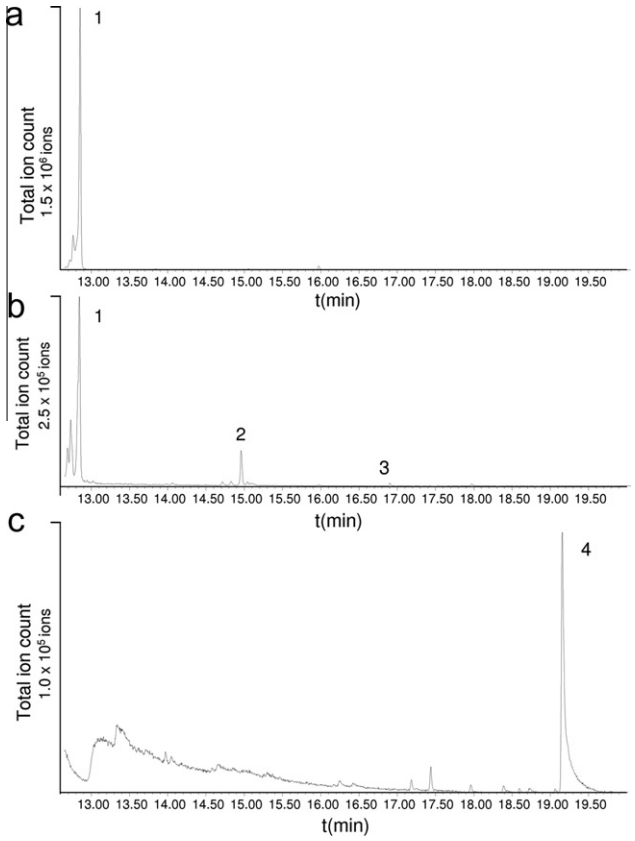


**Fig. 2.** GC–MS analysis of the germacrene A oxidation by CYP71AV8. Co-expression of chicory germacrene A synthase and the CYP71AV8 led to conversion of (1) germacrene A, to (2) germacra-1(10),4,11(13)-trien-12-al, (3) germacra-1(10),4,11(13)-trien-12-ol and (4) germacra-1(10),4,11(13)-trien-12-oic acid. (a) Chromatogram of the *n*-dodecane layer of yeast expressing germacrene A synthase; (b) Chromatogram of the *n*-dodecane layer expressing germacrene A synthase and CYP71AV8; (c) chromatogram of ethyl acetate extract of yeast expressing germacrene A synthase and CYP71AV8.

formation of a side product, an unidentified oxidised sesquiterpene, was also detected (rt = 16.11). Thus it appeared that CYP71AV8 is a valencene oxidase, which preferentially hydroxylates the C2 position of (+)-valencene in the trans-orientation. A small portion of nootkatol is further oxidised to (+)-nootkatone.

### 3.3. Activity of CYP71AV8 on other sesquiterpenes

To further assess the substrate specificity in vivo, the CYP71AV8 was also introduced into yeast strains expressing either germacrene A synthase or amorpha-4,11-diene synthase. Upon co-expression of CYP71AV8 with germacrene A synthase, germacrene A accumulation in the *n*-dodecane layer decreased, while the prevalent novel product was identified as germacra-1(10),4,11(13)-trien-12-al, and a smaller peak of germacra-1(10),4,11(13)-trien-12-ol (Fig. 2). In the ethylacetate extract of the yeast culture germacra-1(10),4,11(13)-trien-12-oic acid was detected after methylation with diazomethane. This suggests that CYP71AV8 catalyzes a three-step conversion of germacrene A to germacra-1(10),4,11(13)-trien-12-oic acid, as was previously shown for the chicory germacrene A oxidase [12].

Co-expression of amorpha-4,11-diene synthase with CYP71AV8 led to partial conversion of produced amorpha-4,11-diene into artemisinic alcohol and artemisinic aldehyde. In addition, small amounts of artemisinic acid and dihydroartemisinic aldehyde were observed. Thus CYP71AV8 can also function as a germacrene A oxidase and an amorpha-4,11-diene oxidase, which preferentially hydroxylates at the allylic C12 position of germacrene A and amorpha-4,11-diene, and can further oxidise the allylic hydroxyl groups into carboxylic acids.

## 4. Discussion

In this work, we describe a novel P450 enzyme from chicory, which can oxidise (+)-valencene at the C2 position, leading to (+)-nootkatone formation. It is the first P450 enzyme that has been demonstrated to be functional in an in vivo production system of valencene-based flavour compounds. Upon expression of CYP71AV8 in a valencene producing yeast strain 68% of the (+)-valencene was oxidised to *trans*-nootkatol, 8% to *cis*-nootkatol and 3% to (+)-nootkatone (Table 1). It is unclear whether the production of (+)-nootkatone results from the activity of CYP71AV8 or the unspecific alcohol dehydrogenase activity present in yeast. The yields of (+)-nootkatone produced per liter yeast culture are still modest. This likely results from the poor production of (+)-valencene and other precursors. Extensive engineering of yeast producing artemisinic acid resulted in production levels of up to 100 mg/L culture [13].

Surprisingly, the same enzyme which oxidises the C2 position in (+)-valencene, also oxidises at the C12 position, when amorpha-4,11-diene or germacrene are supplied as substrates (Fig. 3). This observation may provide novel insights in the regiospecificity of plant P450 enzymes. One of the few reports discussing factors affecting regiospecificity is the pioneering work of Takahashi et al. [10] The authors characterized *H. muticus* premnaspirodiene oxygenase, which introduces an oxygen exclusively in the C2 regioposition of the A ring of both spirodecanes (e.g. premnaspirodiene) and eremophilanes (5-epi-aristolochene, epi-eremophilene and (+)-valencene). In the recently published work by Nguyen et al. [12], the chicory germacrene A oxidase is shown to selectively oxidise at the C12 position of both germacrene A and amorpha-4,11-diene. Thus it would seem that two categories of sesquiterpene modifying P450 enzymes can be distinguished in the plant kingdom: those that act on the A-ring, such as the *H. muticus* premnaspirodiene oxygenase, and those that act on the allylic C12 position, such as the chicory germacrene A oxidase and the *A. annua* amorpha-4,11-diene oxidase. Therefore, finding that a single P450 can oxidise different regio-positions on such structurally related molecules as germacrene A and (+)-valencene was unexpected.

In the past, De Kraker at al. [11], reported that a microsomal extract of chicory could indeed perform both regiospecific reactions. In the latter work, it was postulated that chicory germacrene A oxidase also mediates C-12 oxidation of amorpha-4,11-diene, but that the formation of (+)-nootkatone is catalyzed by a different monooxygenase. This latter mono-oxygenase would possibly catalyse a later step in bitter sesquiterpene lactone biosynthesis, the conversion of costunolide into leucodin. Notably, the authors describe that formation of (+)-nootkatone was inhibited up to 90% by the addition of germacrene A, an effect which would not be in accordance with their two-enzyme hypothesis, but is in accordance with a single cytochrome P450 mediating both (+)-valencene to (+)-nootkatone conversion, as well as germacrene A to germacra-1(10),4,11(13)-trien-12-oic acid conversion. Still, this leaves open the question why two different positions on the sesquiterpene carbon scaffold can be oxidised by the same enzyme. Likely this is the result of a different orientation of (+)-valencene on one hand, and germacrene A and amorpha-4,11-diene on the other hand, in the substrate binding pocket of the P450 enzyme.

Regiospecificity of P450 enzymes is important for the production of fine chemicals. This work clearly shows that regiospecificity of plant P450 enzymes is poorly predictable from the carbon atom

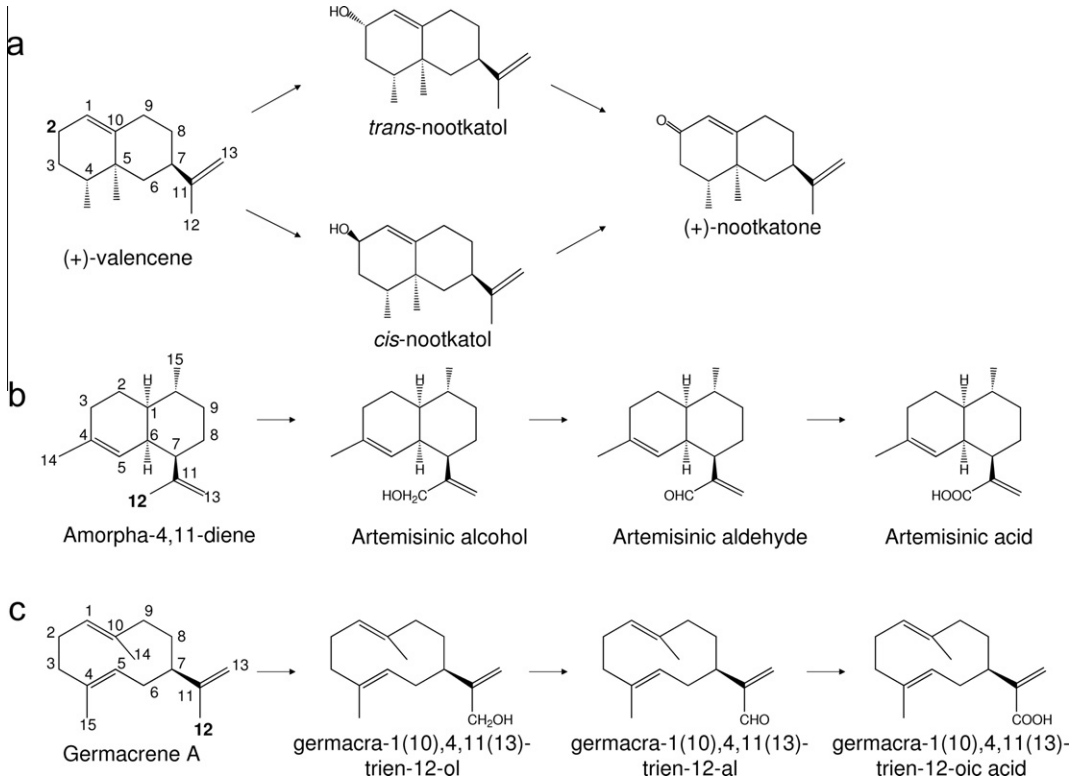


**Fig. 3.** Reactions catalyzed by CYP71AV8. CYP71AV8 catalyses (A) conversion of (+)-valencene to (+)-nootkatone via *trans*- and *cis*- nootkatol, (B) a three-step conversion of germacrene A to germacra-1(10),4,11(13)-trien-12-oic acid, and (C) a three step conversion of amorpha-4,11-diene to artemisinic acid. The preferred position of hydroxylation is marked in bold.

numbering (Fig. 3), which is based on the numbering of the general sesquiterpene precursor farnesyl diphosphate. More knowledge of the three-dimensional properties of the substrate-binding pocket of the P450 enzyme, in addition to more extensive variation of substrates, is needed to fully understand P450 regiospecificity.

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