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REVIEW

A modern purification method for volatile sesquiterpenes produced by recombinant *Escherichia coli* carrying terpene synthase genes

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ABSTRACT

Most volatile sesquiterpenes had been purified from plants using distillation and preparative gas chromatography, which is not applicable to many laboratories that do not possess a needed facility. Thus, this review focuses on a modern purification method for volatile sesquiterpenes using *Escherichia coli* cells that functionally express terpene synthase (*Tps*) genes. It was recently developed that recombinant *E. coli* cells carrying *Tps* genes were cultured in two-layer media (*n*-octane/TB medium) without harming the cells, and the volatile hydrophobic compounds trapped in the *n*-octane were purified by two-phase partition (alkane/alkaline 50% MeOH), silica gel column chromatography, and reversed-phase preparative high-performance liquid chromatography (if necessary). Consequently, it was found that the volatile sesquiterpenes are easily purified, the structures of which can then be determined by nuclear magnetic resonance, $[\alpha]_D$ and gas chromatography–mass spectrometry analyses. The antioxidant activities of several volatile sesquiterpenes are also presented in this review.

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Sesquiterpenes are C15 compounds produced by higher plants via the mevalonate pathway, some of which have floral scents and/or possess pharmaceutical activities. Around the middle of the twentieth century, most volatile sesquiterpenes had been purified from plants using distillation and preparative gas chromatography (GC), and their structures had been determined mainly through their chemical degradation products [1–4]. Since then, distinct purification methods of volatile sesquiterpenes had not been exhibited for some decades. Even after recombinant DNA techniques were generally available, produced sesquiterpenes have often been analyzed with a gas chromatography-mass spectrometry that includes a MS library (GC-MS) without their purification, while their efficient extraction or detection methods were developed (Greenhagen et al., O'maille et al.) [5,6]. At the beginning of 21st century, studies on terpene synthase (TPS) using recombinant *Escherichia coli* (*E. coli*) were undertaken, while they are similarly analyzed with GC-MS without the purification of the TPS products [7–11]. For example, in 2002, Bennet et al. identified lettucenin A, a sesquiterpene lactone biosynthesized via the volatile sesquiterpene germacrene A, as the product of lettuce terpene synthases LTC1 and LTC2 by analyzing the hexane extract of cultured recombinant *E. coli cells* carrying *Tps* genes using a GC-MS library [7]. Furthermore, in 2010, Fujisawa et al. adopted a two-layer culture (*n*-dodecane/culture medium) approach when culturing recombinant *E. coli* carrying *Tps* genes from *Zingiber officinale* and identified (S)-β-bisabolene by analysis of the *n*-dodecane layer containing the produced volatile sesquiterpenes using a GC-MS library [8].

Very few previous studies have addressed methods for purifying the volatile sesquiterpenes produced. In 2008, Agger et al. captured vaporized volatile sesquiterpenes produced during the culturing of *E. coli* carrying *Streptomyces*-derived *Tps* by solid-phase microextraction (SPME) and purified them mainly using preparative octadecyl-silica HPLC. These sesquiterpenes were then identified as (−)-geosmin, germacradienol, pentalene, 2-*epi*-isozizazene, (−)-elemene, (+)-germacrene A, 8a-*epi*-α-selinene, (−)-germacrene D, and β-selinene by ^{1}H nuclear magnetic resonance (NMR) analyses [12]. Furthermore, in 2010, Hu et al. identified the TPS products from *Streptomyces clavuligerus* as (−)-δ-cadinene and (+)-t-muurolol by ^{1}H NMR analyses following their purification by hexane extraction and silica gel chromatography [13]. However, none of the relevant studies emphasized the recovery yields of the produced volatile sesquiterpenes.

In 2008, we began a scientific study to identify volatile sesquiterpenes produced by recombinant *E. coli* carrying product-unidentified *Tps* genes derived from flavor or edible plants. For this study, we needed to establish a method to purify the volatile sesquiterpenes produced with high recovery yields in order to unambiguously

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determine their structures and explore their biological activities. Consequently, we found that a two-layer system with *n*-octane/TB medium can trap the resultant volatile sesquiterpenes in the *n*-octane layer without harming *E. coli* cells, and purified them through two-phase partitioning (*n*-octane + *n*-hexane/alkaline 50% MeOH), silica gel column chromatography, and reversed-phase preparative high-performance liquid chromatography (HPLC) (if necessary) [14–17]. We further demonstrated the usefulness of the developed purification method of volatile sesquiterpenes for determining their absolute structures, *i.e.* their structures were confirmed using NMR, $[\alpha]_D$, and GC-MS analyses, exhibiting their physicochemical data, which were previously unreported in many cases [14–17]. In addition, we evaluated the antioxidant activities of several of the purified volatile sesquiterpenes. We report these results in this review.

Tps genes that are introduced in this review [14–17]

Tps genes that are introduced in this review were isolated from the flowers of the genera *Humulus* (*HILIS/NES*) and *Camellia* (*CbTps1* and *ChTps1*), the rhizomes of the genus *Zingiber* (*ZzZss2* (*ZSS2*) and *ZoTps1*), and the young leaves of the genus *Aralia* (*AcTps1, AcTps2,* and *AeTps1*), which have been described [14,15] or will soon be submitted [16,17]. These isolated *Tps* genes (usually the full length cDNAs) were individually transferred into a mevalonate-pathway-engineered *E. coli* using the *E. coli* expression vector pETDuet-1 (Merck) without their codon-usage optimization. The mevalonate-pathway-engineered *E. coli* was prepared by introducing plasmid pAC-MeV/Scidi/Aacl into *E. coli* Bl21 (DE3) [18,19]. This plasmid contains the mevalonate-pathway gene cluster from *Streptomyces* sp. strain CL190, which consists of six enzyme genes mediating the conversion from acetoacetyl-CoA to isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP), the *Saccharomyces cerevisiae* IPP isomerase (type 1) gene (*Scidi*), which is expected to increase the metabolic flow from DMAPP to farnesyl diphosphate (FPP), and the acetoacetate-CoA ligase gene (*Aacl*) for the utilization of Li acetoacetate (LAA) as the substrate by generating acetoacetyl-CoA [18,19].

Production of volatile sesquiterpenes in the culture of recombinant *E. coli* [14–17]

Each recombinant *E. coli* was introduced to the TB medium (100 mL/500 mL Erlenmeyer flask) containing 1 mM ampicillin (AP) and chloramphenicol (Cm), and cultured at 30 °C with 150 rpm agitation until the optical density (OD) of the culture at 600 nm reached 1.0. A 2 mL aliquot of the culture was then transferred to a 500 mL Sakaguchi flask containing 100 mL of TB medium with 1 mM Ap, 1 mM Cm, 1 mM isopropyl thiogalactoside, and 100 mM LAA. Then, 20 mL *n*-octane was added to the flask and the mixture was cultured at 20 °C for 48 h. The culturing of recombinant *E. coli* was performed at the 1 L (10 flasks) or 2 L (20 flasks) scale according to the amount of products required. Under these culture conditions, the produced volatile sesquiterpenes were trapped in the *n*-octane layer.

In previous studies, *n*-dodecane was used for the two-phase (alkane/medium) culturing of recombinant *E. coli* carrying *Tps* genes to trap the produced volatile sesquiterpenes. However, we used *n*-octane for this purpose because, like *n*-dodecane, *n*-octane is non-toxic to recombinant *E. coli*, but it can be removed under reduced pressure using a diaphragm pump, allowing the volatile sesquiterpenes in the *n*-octane to be concentrated easily.

Purification of the produced volatile sesquiterpenes [14–17]

The *n*-octane layer (200 mL) of a 1 L culture was separated from the 2-YT medium in a separation funnel and then added to *n*-hexane (200 mL). The alkane layer (*n*-octane + *n*-hexane, 400 mL) was washed with alkaline 50% MeOH (400 mL) twice to remove any free fatty acids produced by the host *E. coli*. It was then concentrated to 3–5 mL *in vacuo*. The produced volatile sesquiterpenes in the alkane layer were monitored by silica gel thin-layer chromatography (TLC) (Merck) developed with *n*-pentane or *n*-pentane-EtOAc (5:1) and visualized as blue spots with molybdophosphoric acid color development [15]. In the silica gel TLC analyses developed with *n*-pentane, hydrocarbon volatile sesquiterpenes showed Rf values of 0.5–0.8, and volatile sesquiterpenes with OH functionalities showed Rf ~0. In those developed with *n*-pentane-EtOAc (5:1), hydrocarbon volatile sesquiterpenes showed Rf > 0.9, and volatile sesquiterpenes with OH functionalities showed Rf values of 0.3–0.5.

The concentrated alkane layer was applied to a silica gel (Silica Gel 60, Kanto Chemical) column (20 i.d. × 200 mm) filled with *n*-pentane and eluted with *n*-pentane (200 mL) and then *n*-pentane-EtOAc (5:1) (200 mL) stepwisely. The eluate was collected in 7 mL fractions (*n*-pentane fractions: 1–30; *n*-pentane-EtOAc (5:1) fractions: 31–60). The hydrocarbon volatile sesquiterpenes were eluted in the *n*-pentane fractions and the volatile sesquiterpenes with OH functionalities were eluted in the *n*-pentane-EtOAc (5:1) fractions. All of the fractions were checked using silica gel TLC developed with *n*-pentane (fractions 1–30) or *n*-pentane-EtOAc (5:1) (fractions 31–60), and the fractions containing individual sesquiterpenes were combined, concentrated to dryness (100 mm Hg), and analyzed using ^{1}H NMR in $CDCl_3$. The concentration step was performed under moderate vacuum conditions (100 mm Hg) as to not vaporize the volatile sesquiterpenes.

When the fractions contained mixtures of sesquiterpenes, we performed preparative octadecyl-silica (ODS) HPLC for further purification [column: C30-UG-5 (10 i.d. × 250 mm, Developsil) or ADME (10 i.d. × 250 mm, Shiseido); solvent: 80–95% CH_3CN (isocratic); flow rate: 3.0 mL/min; diode array detection (200–300 nm)]. Using this ODS HPLC, each sesquiterpene was eluted as a pure compound within 30 min. Each eluted peak solution was diluted with the same volume of water and double the volume of *n*-pentane before shaking in a separation funnel. The *n*-pentane layer (upper layer) containing the volatile sesquiterpene was collected, dried over Na_2SO_4 for 30 min, and concentrated to dryness (100 mm Hg) to give a pure volatile sesquiterpene.

This purification method is summarized in Figure 1. The yield (mg) of each purified volatile sesquiterpene from 1 L culture (10 flasks) was shown in Figure 2.

Structural analyses of the purified volatile sesquiterpenes [14–17]

The structures of the purified volatile sesquiterpenes were analyzed by 1D (^1H and ^{13}C NMR) and 2D (^1H–^1H DQF COSY, HMQC, HSQC-TOCSY, HMBC, and NOESY) NMR spectroscopy in $CDCl_3$, and their planar structures were completely determined (except those of germacrene A and hedycaryol). HSQC-TOCSY analyses are especially effective here because each ^1H–^1H vicinal spin network can be assigned completely using the cross peaks from isolated (i.e. non-overlapped) ^{13}C signals. Since the ^1H signals of germacrene A and hedycaryol are very broad owing to their structural tautomerism, their identifications were performed using GC-MS analyses followed by comparison with library data. Some of their relative configurations were analyzable by NOESY spectroscopy, and their absolute configurations were determined from $[\alpha]_D$ values and GC-MS analyses (matching with library data).

Identification of volatile sesquiterpenes produced by recombinant *E. coli* [14–17] and their antioxidant activities

We purified and identified (3*R*)-nerolidol (*HILIS/NES*) and (3*R*)-linalool (monoterpene) (*HILIS/NES*) from *Humulus* (Figure 2(A)), hedycaryol (*CbTps1*) and valerianol (*ChTps1*) from *Camellia*, (Figure 2(B)), β-eudesmol (*ZzZss2* (*ZSS2*)) and β-bisabolene (*ZoTps1*) from *Zingiber* (Figure 2(C)), germacrene A (*AcTps1* and *AcTps2*), β-panasinene (*AcTps2*), 2-epi-β-caryophyllene (*AcTps2*), α-humulene (*AcTps2*), α-copaene (*AeTps1*), δ-cadinene (*AeTps1*), β-cubebene (*AeTps1*), and germacrene D (*AeTps1*) from *Araliaceae* (Figure 2(D)).

Additionally in this review, we evaluated the antioxidant activities of some of the purified sesquiterpenes against rat brain homogenate lipid peroxidation [20], and reported them for the first time. The activities are shown as IC_{50} values, i.e. the concentration that results in 50% inhibition (μM), in Table 1. δ-Cadinene was proved to exhibit potent antioxidant activity in this study. Further studies on the antioxidant activity of δ-cadinene are currently in progress.

Conclusions

The purification method presented in this article is commonly available in modern chemical laboratories and is applicable not only to the volatile sesquiterpenes produced in the culture of recombinant *E. coli* carrying *Tps* genes, but also to those isolated from flavor plants

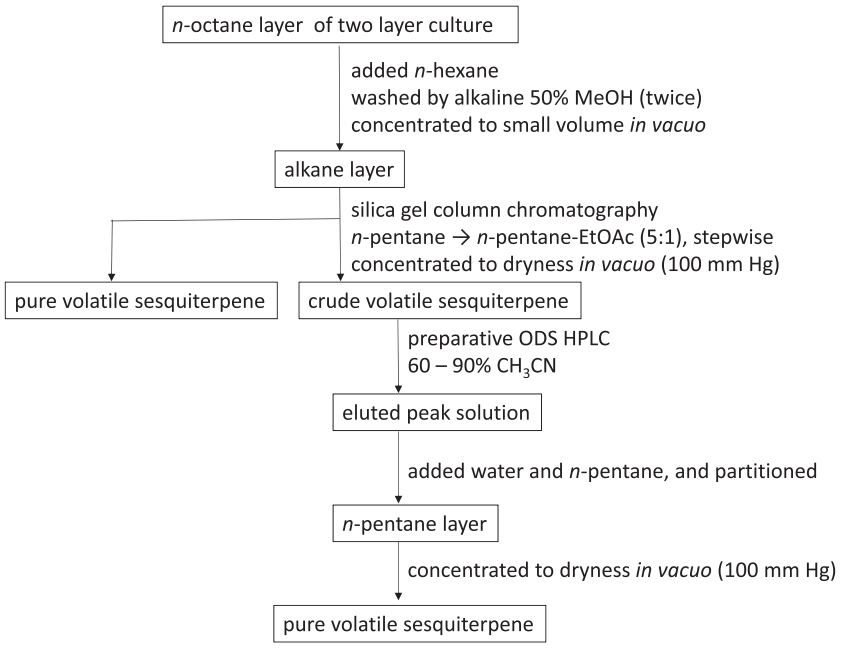

Figure 1. Purification method for volatile sesquiterpenes.

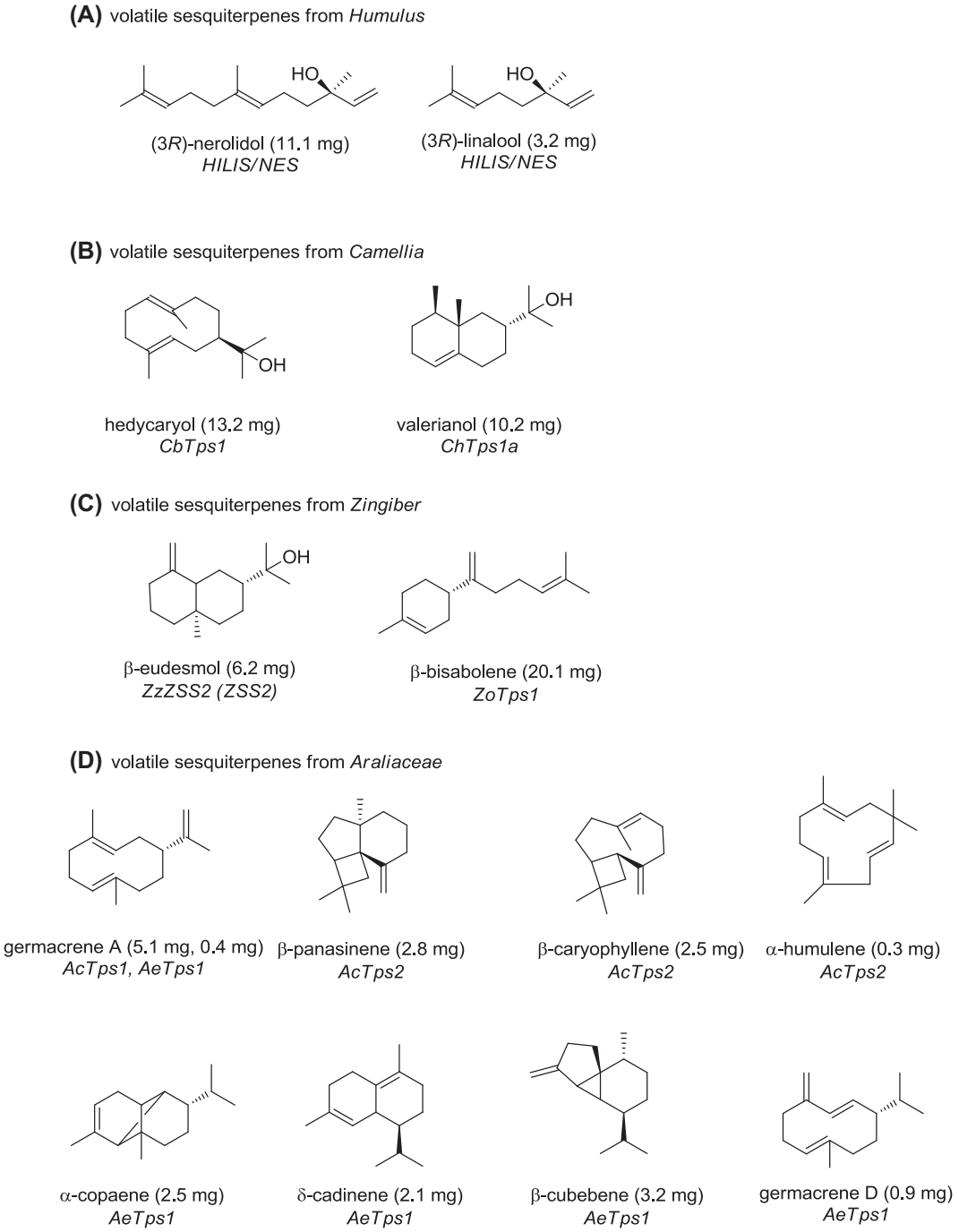

Figure 2. The structures of volatile sesquiterpenes produced by *Tps* genes belonging to the families *Humulus* (A), *Camellia* (B), *Zingiber* (C), and *Araliaceae* (D). The yield (mg) of purified volatile sesquiterpene from 1 L culture was shown in parenthesis.

Table 1. Antioxidant activities of volatile sesquiterpenes.

Compound	IC_{50} (μM)
(3*R*)-nerolidol	>200
Valerianol	180
β-bisabolene	>200
Germacrene A	50
β-caryophyllene	24
α-copaene	36
δ-cadinene	3.2
β-cubebene	32
Germacrene D	34
Catechin (positive control)	4.0

(after distillation or extraction with an organic solvent). We were able to analyze and unambiguously identify the structures of the purified volatile sesquiterpenes using NMR, $[\alpha]_D$, and GC-MS. Furthermore, various studies, including biological activity assessment, are possible using the purified volatile sesquiterpenes. We are currently in the process of assessing their biological activities, and the results will be reported in due course.

We hope that the purification method established here will be adopted by many researchers involved in the isolation of volatile sesquitepenes.

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Disclosure statement

No potential conflict of interest was reported by the author.

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