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Detection of the Previously Unobserved Stereoisomers of Thujone in the Essential Oil and Consumable Products of Sage (*Salvia officinalis* L.) Using Headspace Solid-Phase Microextraction−Gas Chromatography−Mass Spectrometry

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Supporting Information

ABSTRACT: The discovery of the (+)-α-thujone and (−)-β-thujone stereoisomers in the essential oil of sage (*Salvia officinalis* L.) and dietary supplements is documented for the first time. The detection was accomplished using a chiral resolution protocol of racemic α-/β-thujone on headspace solid-phase microextraction−gas chromatography−mass spectrometry. Because the previously unreported stereoisomers, (+)-α-thujone and (−)-β-thujone, are not commercially available, a three-step synthesis of racemic thujone from commercially available starting materials was developed. Thermolysis studies demonstrated that no racemization at the cyclopropane stereocenters occurs, corroborating that the detection is not an artifact from the hydrodistillation process. The developed chiral resolution of thujone was also used to provide evidence for the absence of the (+)-α-thujone and (−)-β-thujone enantiomers in other common thujone-containing essential oils.

KEYWORDS: *thujone, chiral GC−MS, Salvia officinalis L., HS-SPME−GC−MS, essential oils*

INTRODUCTION

Thujone is a monoterpene natural product that is present in the essential oils of many common plants, including sage, cedarleaf, and wormwood. Thujone is a neurotoxin that modulates the γ-aminobutyric acid (GABA)-gated chloride channel[1] and causes convulsions in acute exposure and unconsciousness and death in rare examples.[2,3] The estimated allowable daily intake for thujone is roughly 3−7 mg/day for a duration of no more than 2 weeks.[3] The known diastereomers of thujone have been shown to have different toxicity, with (−)-α-thujone being reported as more toxic than the (+)-β diastereomer.[1] To date, the presence of thujone in plants appears to be limited to various ratios of solely the (−)-α and (+)-β stereoisomers.[4−13]

Thujone has four potential stereoisomers as a result of the methyl and cyclopropyl stereocenters of the bicyclo[3.1.0]-hexanone core structure, as illustrated in Figure 1. The previously reported stereoisomers are diastereomers that are epimers at the methyl substituent with identical stereochemistry at cyclopropane. The unreported stereoisomers, (+)-α- and (−)-β-thujone, are each the enantiomers of the known diastereomers that possess the opposite stereochemistry at cyclopropane. The biosynthetic pathway for the common (−)-α and (+)-β stereoisomers has been proposed for several plants. In *Thuja plicata*[14] and *Salvia officinalis* L.,[15] the common stereoisomers are proposed to be derived from (+)-sabinone, as highlighted in Figure 2. The reported thujone stereoisomers obtain their stereochemical assignment at the cyclopropane ring from these precursors, while the methyl stereochemistry varies based on the selectivity of the hydrogenation step.

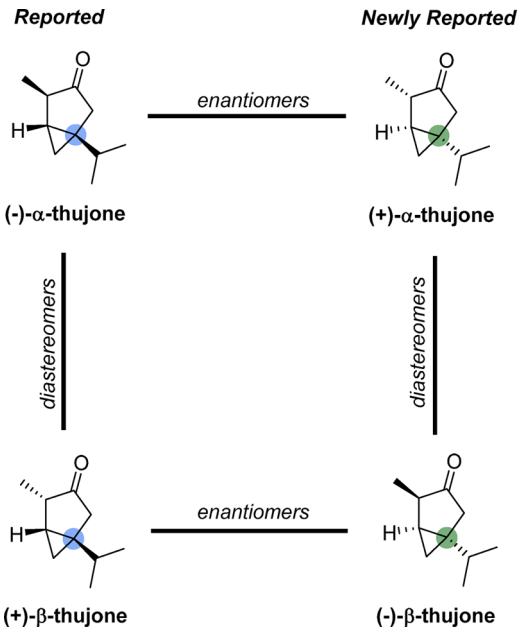

Figure 1. Possible stereoisomers of thujone.

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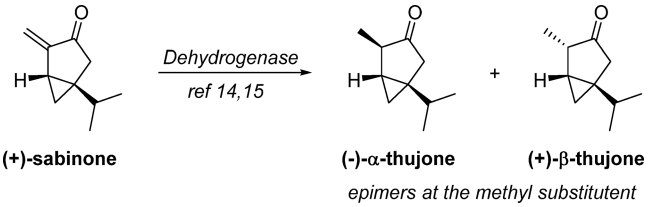

Figure 2. Proposed biosynthetic origin of the stereochemistry in the reported stereoisomers of thujone.

An abundance of research exists on the presence of (−)-α- and (+)-β-thujone in plants;[4−13] however, none of these studies addresses the possible presence of the corresponding stereoisomers, (+)-α- and (−)-β-thujone. While many plants produce single enantiomers of natural products, the enantiomeric compositions of certain phytochemicals can vary greatly based on a variety of factors,[16] including harvest time, geographical origin, and phenotype.[17,18] For example, limonene exists almost exclusively as the *R* enantiomer in most citrus oils[19] and as the *S* enantiomer in the oil of *Abies alba*,[20] while β-elemene is found as a mixture of enantiomers in *Scapania nemorea*, exclusively the (+) enantiomer in *Piper nigrum*, and 97% of the (−) enantiomer in the oil in *Barbilophozia barbata*.[21]

Chiral gas chromatography−mass spectrometry (GC−MS) is the most popular method for the enantiodetermination of volatile phytochemicals. This enantiomeric analysis of the chiral components in essential oils can be useful as a fingerprint for authenticating the species, cultivar, or area of origin of the sample. It can also be used to determine if the essential oil has been adulterated.[22,23] Because the chiral phase and temperature parameters of GC−MS can have a dramatic effect on the ability to separate chiral analytes,[24] the racemate is typically required as the standard for method development. Once the enantiomers have been resolved, the identification and quantitation of chiral terpenes in essential oils becomes feasible. A common barrier to this type of analysis is the lack of necessary enantiomers of the phytochemicals to accomplish the chiral resolution. This is the case with thujone, where this analysis likely has been hindered as a result of the unavailability of the unreported stereoisomers. This is the case with thujone, where this analysis likely has been hindered as a result of the unavailability of the unreported stereoisomers. In these instances, organic synthesis can play an important role in providing the requisite natural products.[20]

Herein, the common essential oils known for their high thujone content were investigated to determine whether the unreported stereoisomers of the monoterpene are present. The study focused primarily on garden sage because it is a popular thujone-containing medicinal tea and supplement. Additionally, the thujone concentration and ratio of α/β stereoisomers are known to vary widely in *S. officinalis* L. based on several factors,[25] including climate,[26] developmental stage,[27] and season,[8] that may correspond to an analogous enantiomeric variation. To this end, a three-step synthesis of racemic thujone was developed for the purpose of the chiral resolution of the stereoisomers for GC−MS analysis. Commercial essential oils, herbal teas, spices, and dietary supplements were then analyzed for the presence of all four stereoisomers of thujone.

MATERIALS AND METHODS

Chemicals. All chemicals and solvents (reagent grade) were purchased from Sigma-Aldrich, Inc. (St. Louis, MO) and employed without further purification. Some sage oils and thujone standards were purchased from Sigma-Aldrich, Inc. (St. Louis, MO), including (−)-α-thujone and the thujone standard mixture.

Essential Oils. The essential oils were reported to be isolated by steam distillation and are listed with sample ID, essential oil, distributor, and country of origin in the Supporting Information.

Solid-Phase Microextraction (SPME) Sample Preparation. Samples were placed in headspace (HS) vials (20 mL) sealed with polytetra uoroethylene (PFTE)/silicone septum caps (Supelco, Bellefonte, PA, 27236) with either 5 L of the essential oil, 2.0 g of loose tea (the contents of approximately two teabags), or 2.0 g of the spice or dietary supplement (the contents of approximately 15−20 capsules).

SPME and Chromatography. A 1 cm triple-phase 50/30 m divinylbenzene (DVB)/carboxen/polydimethylsiloxane (PDMS)-coated fiber was employed (Supelco, Bellefonte, PA). The literature revealed that this coating is the best solution for the extraction of volatiles present in sage.[28] Prior to first use, fiber was conditioned according to the recommendations of the supplier of 300 °C for 60 min. The thujone enantiomers were extracted and detected using the following instruments and methodology: a MPS2 autosampler equipped with Maestro1 software, version 1.4.8.14/3.5 (Gerstel, Baltimore, MD), and two Agilent 7890A gas chromatographs, one coupled to an Agilent 5975C mass selective detector for qualitative analysis and a second Agilent 7890A gas chromatograph coupled to a ame ionization detector for semi-quantitative analysis. To ensure no carryover of analytes, the fiber was thermally cleaned between analyses using the default temperature program.

The HS was extracted for 2 min at 21 °C for the essential oils, 15 min for the spices and teas, and 60 min for the dietary supplements. Bound volatiles were desorbed for 5 min at 250 °C in the injector operated in split mode (split ow, 50 mL/min; split ow, 20 mL/min for spices, teas, and capsules). Volatile compounds were separated using fused silica capillary columns, 30 m × 0.25 mm, 0.25 m film thickness, with three different phases: Restek Rt-βDEXsa (Restek, Bellefonte, PA), Restek Rt-βDEXsm (Restek, Bellefonte, PA), and Agilent Cyclosil-B. Helium was used as the carrier gas at a constant ow rate of 1.0 mL/min. The racemic thujone standard was resolved using an oven temperature program of 60 °C for 3 min and then 5 °C/min to 170 °C for 6 min. To prevent co-elution of camphor and linalool present in sage essential oils, teas, spices, and capsules, a slightly different temperature program of 120 °C for 15 min and then 5 °C/min to 175 °C was used. The MS settings for all analysis were as follows: transfer line, 250 °C, source temperature, 230 °C; and SCAN mode, 40−200 Da, with a 0.3 s scan interval and a 2 min solvent delay. Mass spectra were recorded following electronic impact ionization at 70 eV. Data were collected and analyzed by GC−MS Agilent ChemStation software (GCMSD ChemStation E02.01 with enhanced data analysis) using the NIST-14 mass spectral database. The percentages of each component are reported as raw percentages based on total ion current without standardization.

Synthesis of Racemic α-/β-Thujone. *General Experimental Procedures.* Proton and carbon nuclear magnetic resonance (^{1}H and ^{13}C NMR) spectra were recorded at 400 and 600 and 150 MHz, respectively, with solvent resonance as the internal standard (^{1}H NMR, $CDCl_3$ at 7.26 ppm; ^{13}C NMR, $CDCl_3$ at 77.0 ppm). ^{1}H NMR data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; dd, doublet of doublets; t, triplet; q, quartet; and m, multiplet), coupling constants (Hz), and integration. Thin-layer chromatography (TLC) visualization was accomplished with ultraviolet (UV) light and/or either aqueous potassium permanganate ($KMnO_4$) or aqueous ceric ammonium molybdate (CAM) solution, followed by heating. All reactions were carried out under an atmosphere of nitrogen in oven-dried glassware with magnetic stirring. Yield refers to the isolated yield of analytically pure material. Yields are reported for a specific experiment and, as a result, may differ slightly

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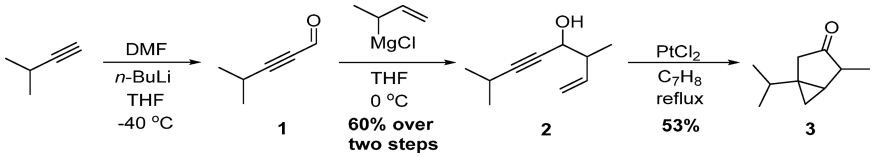

Figure 3. Synthesis of racemic thujone.

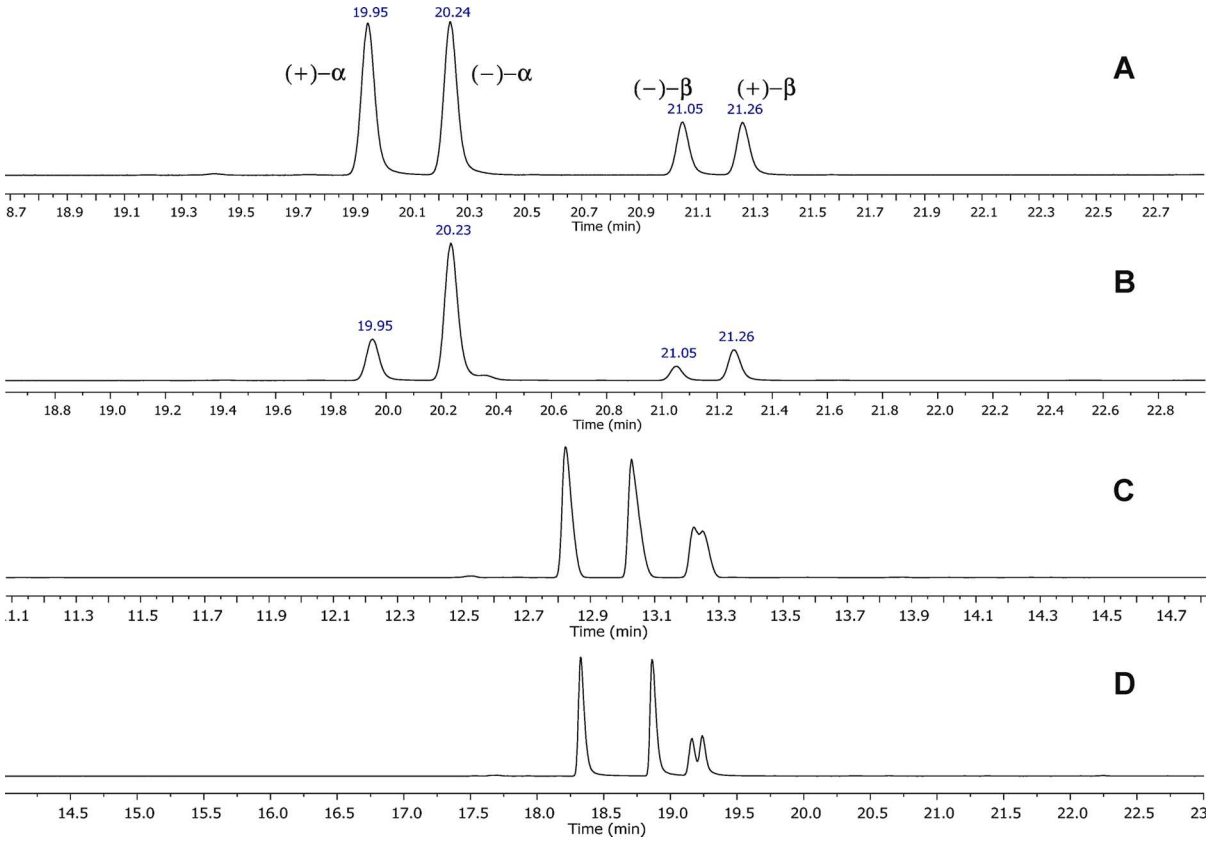

Figure 4. Extracted chromatograms for the chiral GC−MS resolution of the four thujone stereoisomers. (A) Baseline chiral resolution of the four enantiomers of thujone from the synthetic sample on a Rt-*β*DEXsa column. (B) Synthetic sample on a Rt-*β*DEXsa column spiked with commercially available (−)-*α*-thujone and (+)-*β*-thujone samples. (C) Synthetic sample on a Rt-*β*DEXsm column. (D) Synthetic sample on an Agilent Cyclosil-B.

from those found in Figure 3, which are averages of at least two experiments.

*Preparation of 4-Methylpent-2-ynal (**1**).* A solution of dry tetrahydrofuran (THF, 15 mL) and 3-methyl-1-butyne (1.0 mL, 10 mmol, 1.0 equiv) was added to a ame-dried ask and cooled to −40 °C. A 2.2 M solution of *n*-butyl lithium in THF (5.0 mL, 11 mmol, 1.1 equiv) was added dropwise over 2 min and then stirred for 5 min. Anhydrous dimethylformamide (DMF, 1.50 mL, 20 mmol, 2.0 equiv) was then added to the ask in one portion, and the ice bath was removed. The solution was allowed to warm to room temperature and stirred for an additional 30 min. The reaction was quenched by slow addition to a vigorously stirred mixture of 0.6 M KH_2PO_4 (50 mL) and Et_2O (30 mL) at 0 °C. The resulting mixture was stirred for 10 min. The layers were separated, and the aqueous layer was extracted with diethyl ether (3 × 10 mL). The organic extracts were combined, washed with water and then with brine (20 mL), dried with $MgSO_4$, and concentrated *in vacuo*. As a result of the volatility of the product, the solvent was removed *in vacuo* in an ice bath to afford 4-methylpent-2-ynal as a yellow oil. The product was taken on crude to the next step. TLC (10% EtOAc/hexanes) R_f = 0.68. ^{1}H NMR spectral data matched those reported for the title compound.[29]

*Preparation of 3,7-Dimethyloct-1-en-5-yn-4-ol (**2**).* A 0.5 M solution of 1-methyl-2-propenylmagnesium chloride (40 mL, 20 mmol, 2.0 equiv) was added to a ame-dried ask and cooled to 0 °C. The crude 4-methylpent-2-ynal (**1**) was then added dropwise, and the solution was stirred at 0 °C for 1 h. The reaction was then quenched at 0 °C with saturated ammonium chloride (20 mL) and stirred for 10 min. The layers were separated, and the aqueous layer was extracted with diethyl ether (3 × 10 mL). The organic extracts were combined, washed with brine (20 mL), dried with $MgSO_4$, and concentrated *in vacuo*. Purification by ash chromatography (10% EtOAc/hexanes) yielded 910 mg (60% over two steps) of the product as a colorless oil as a mixture of diastereomers. Analytical data for compound **2**: Major diastereomer: ^{1}H (500 MHz, $CDCl_3$) : 5.89−5.80 (m, 1H), 5.17−5.13 (m, 2H), 4.26−4.24 (m, 1H), 2.60−2.57 (m, 1H), 2.44−2.40 (m, 1H), 1.85 (d, *J* = 7.2 Hz, 1H), 1.17 (d, *J* = 6.8 Hz, 6H), 1.10 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) : 139.6, 116.4, 92.0, 78.6, 66.3, 44.7, 23.0, 15.3. Minor diastereomer: ^{1}H (500 MHz, $CDCl_3$) : 5.89−5.80 (m, 1H), 5.17−5.13 (m, 2H), 4.20−4.18 (m, 1H), 2.60−2.57 (m, 1H), 2.44−2.40 (m, 1H), 1.84 (d, *J* = 5.2 Hz, 1 H), 1.17 (d, *J* = 6.8 Hz, 6H), 1.12 (d, *J* = 6.8 Hz, 3H). ^{13}C NMR (150 MHz, $CDCl_3$) : 139.1, 116.8, 92.1, 78.3, 66.1, 44.5, 20.5, 15.6. TLC (20% EtOAc/hexanes) R_f = 0.58. HRMS (ESI^-) calcd for $C_{10}H_{16}O$ + $HCOO^-$, 197.1183; found, 197.1185.

*Preparation of Racemic Thujone (**3**).* Propargyl alcohol (**2**, 200 mg, 1.314 mmol, 1 equiv) and dry toluene (7.0 mL) were added to a ame-dried ask ushed with nitrogen. $PtCl_2$ (52 mg, 0.197 mmol, 0.15 equiv) was added, and the reaction was stirred at re ux until the reaction was complete by TLC (36 h). The reaction was cooled to room temperature and quenched with aqueous ammonium chloride

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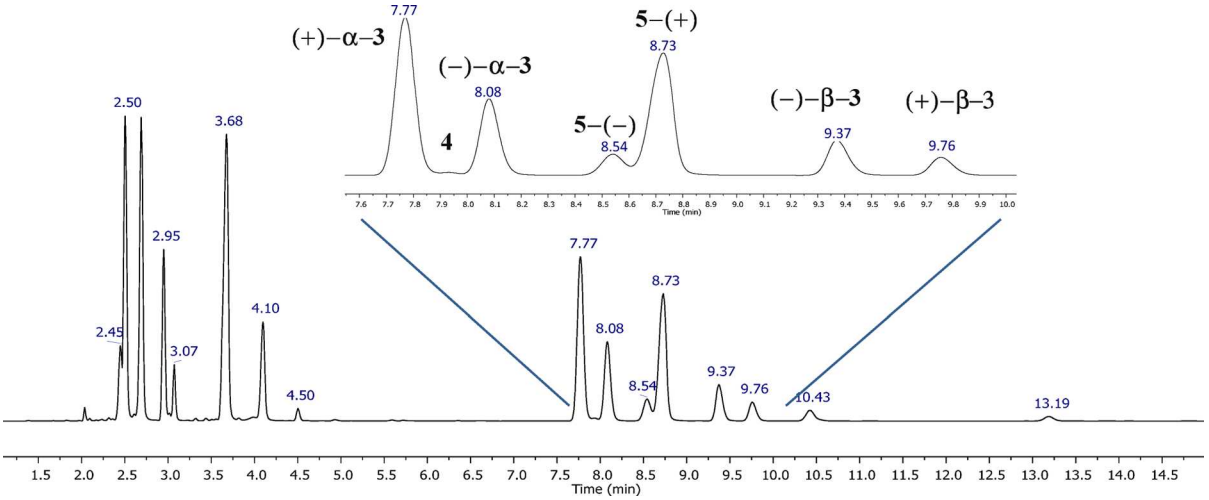

Figure 5. Representative sample of sage (BB) containing all four stereoisomers with the extracted chromatogram, illustrating the thujone stereoisomers (**3**) with linalool (**4**) and camphor (**5**).

(15 mL). The layers were separated, and the aqueous layer was extracted with diethyl ether (3 × 10 mL). The organic extracts were combined, washed with brine (15 mL), dried with $MgSO_4$, and carefully concentrated *in vacuo*. Purification by ash chromatography (5% EtOAc/hexanes) afforded 110 mg (52%) of the product as an amber oil with a 2:1 diastereomeric ratio (α/β). The ^1H and ^{13}C spectral data matched those reported for thujone.[30] TLC (5% EtOAc/hexanes) R_f = 0.47.

RESULTS AND DISCUSSION

Synthesis. To accomplish the chiral resolution necessary for enantiodetermination, a racemic sample of the natural product was required. To date, only one total synthesis of thujone has been reported by Oppolzer and co-workers.[31] This 10 step diastereoselective synthesis of (−)-α-thujone uses a palladium-

Table 1. Percent Concentrations of α-/β-Thujone in the Volatile Portion of the Essential Oil Samples Not Containing the Unreported Stereoisomers

entry[a]	essential oil[b]	(−)-α (%)	(+)-β (%)
EO1	*Thuja occidentalis*	43.0	8.3
EO2	*Thuja occidentalis*	86.7	9.6
EO3	*Achillea fragrantissima*	33.1	6.9
EO4	*Artemisia vulgaris*	37.4	6.7
EO5	*Artemisia vulgaris*	8.0	2.2
EO6	*Artemisia vulgaris*	57.7	8.6
EO7	*Artemisia vulgaris*	6.9	3.6
EO8	*Artemisia herba-alba*	67.3	7.1
EO9	*Artemisia herba-alba*	13.0	3.1
EO10	*Artemisia herba-alba*	59.8	7.6
EO11	*Artemisia herba-alba*	62.0	8.2
EO12	*Tanacetum vulgare*	1.2	92.0
EO13	*Salvia officinalis*	15.9	1.5
EO14	*Salvia officinalis*	15.9	1.6
EO15	*Salvia officinalis*	19.5	2.2
EO16	*Salvia officinalis*	19.5	4.6
EO17	*Salvia officinalis*	20.2	3.0
EO18	*Salvia officinalis*	22.6	2.6
EO19	*Salvia officinalis*	18.3	2.5
EO20	*Salvia officinalis*	3.2	2.0

[a]Mean values of triplicate analysis. [b]The presence of the unreported stereoisomers was not detected.

Table 2. Enantiomeric Composition of α-/β-Thujone in Samples of *S. officinalis* Essential Oil

entry[a]	(+)-α	(−)-α	(−)-β	(+)-β
A	0.3	17.6	0.1	4.7
B	2.9	12.8	2.6	1.4
C	2.9	15.9	0.7	3.4
D	4.1	8.5	1.1	1.1
E	4.6	8.6	1.0	1.1
F	11.1	10.9	2.4	1.2
G	11.3	0.3	2.5	0.9
H	13.1	6.4	3.2	1.8
I	14.3	3.2	3.1	0.7
J	18.0	5.6	4.3	1.5
K	12.3	3.7	2.6	1.4
L	16.5	3.4	3.8	0.6
M	16.2	0.2	3.6	1.3
N	15.4	3.2	3.4	1.1
O	16.7	3.2	3.8	0.6
P	15.7	3.2	3.5	0.6
Q	10.1	22.9	2.3	2.4
R	16.2	3.0	3.7	1.0
S	11.3	0.3	2.5	0.9
T	5.2	14.0	2.4	1.9
U	6.9	12.3	1.6	1.8

[a]Mean values of triplicate analysis.

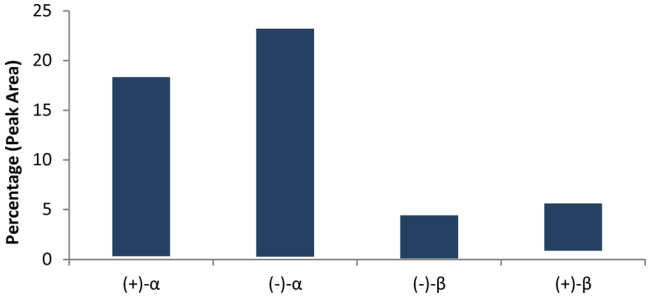

Figure 6. Comparison of upper and lower limits of the four stereoisomers in thujone samples containing the unreported stereoisomers (A−U).

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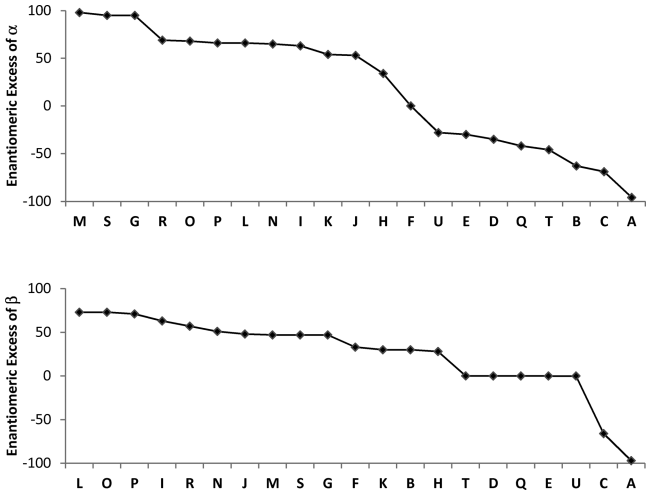

Figure 7. Enantiomeric excess for the essential oils containing the unreported stereoisomers. Positive ee represents the unreported enantiomer as the major enantiomer. (Top) Analysis for α-thujone. (Bottom) Analysis for β-thujone.

Table 3. Enantiomeric Composition of α-/β-Thujone in Various Dietary Supplements (DS), Spices (S), and Teas (T)

entry[a]	(+)-α	(−)-α	(−)-β	(+)-β
DS1	17.4	4.1	5.1	2.1
DS2	25.0	5.7	6.0	2.0
DS3	nd	3.2	nd	2.0
DS4[b]	nd	nd	nd	nd
DS5[c]	nd	nd	nd	nd
S1	nd	23.0	nd	8.6
S2	nd	30.6	nd	9.5
S3	nd	7.7	nd	1.8
S4	nd	27.4	nd	10.5
S5	nd	0.3	nd	0.9
S6	nd	21.9	nd	8.7
T1	nd	42.0	nd	8.2
T2	nd	26.6	nd	13.5
T3	nd	0.3	nd	0.2
T4	nd	30.1	nd	11.5
T5	nd	4.0	nd	2.1
T6	nd	8.9	nd	4.0

[a]Mean values of triplicate analysis. [b]Contains 60% camphor. [c]Contains 71% furfural.

catalyzed intramolecular cyclization of propargyl carbonates to form the carbon skeleton of monoterpene. Because a racemic synthesis was desired, we sought to develop a more concise, scalable synthesis of *rac*-thujone. The proposed synthesis relies on the methodology developed by Fürstner and co-workers for the cycloisomerization of hydroxylated enynes.[32] This Pd-catalyzed formation of bicyclo[3.1.0]hexanone derivatives was used by Fürstner and co-workers to synthesize a related natural product, sabinone.

The synthesis, illustrated in Figure 3, commenced with the formylation of 3-methyl-1-butyne with DMF to provide ynal (**1**).[29] As a result of the volatility of aldehyde, compound **1** was taken to the next step without purification. The addition of 1-methyl-2-propenylmagnesium bromide to the crude compound **1** installed the full carbon framework for thujone as alcohol (**2**) in a 60% yield over two steps. While the original report was able to use a catalyst loading of 5 mol %, this substrate required a higher catalyst loading of 12 mol % and a higher temperature of 110 °C. The platinum-catalyzed cycloisomerization using modified conditions of Fürstner and co-workers then provided racemic thujone in a 53% yield and 1:1 diastereomeric ratio.

Chiral Resolution of the Thujone Enantiomers. With the racemic sample of thujone in hand, an evaluation of the feasibility of three chiral columns to resolve the thujone stereoisomers was investigated and is illustrated in Figure 4. As depicted in Figure 4A, SPME−GC−MS on a Rt-βDEXsa column provides clear baseline separation for all four enantiomers. The enantiomeric elution order was determined by spiking with commercially available (−)-α-thujone and (+)-β-thujone, which provides a roughly 9:1 mixture of the α/β enantiomer, respectively. The corresponding increase in the analyte concentration verified the identity of the four enantiomers, as illustrated in Figure 4B. The retention times for (+)-α, (−)-α, (−)-β, and (+)-β stereoisomers were 19.95, 20.24, 21.05, and 21.26 min, respectively, with resolution factors of 2.73 for the α enantiomers and 2.30 for the β enantiomers. To our knowledge, this is the first method for the chiral resolution of all of the enantiomers of thujone. The Rt-βDEXsm (Figure 4C) and Agilent Cyclosil-B (Figure 4D) provided poorer resolution.

Analysis of Thujone-Containing Essential Oils. On the basis of the chiral resolution described above, the HS-SPME−GC−MS method was applied to a series of thujone-containing essential oils from various suppliers. The original chiral resolution protocol was insu cient for fully separating the analytes of the essential oils. To account for the presence of camphor, linalool, and borneol (retention time of 10.43 min), the temperature program had to be modified to provide baseline separation of the thujone stereoisomers (**3**), linalool (**4**), and the two enantiomers of camphor (**5**), as illustrated in Figure 5. The identity and stereochemistry of the peaks for camphor and linalool were elucidated by spiking with pure standards. A total of 41 commercial essential oils were tested for the presence of the unreported stereoisomers of thujone, with the semi-quantitative results reported in Tables 1 and 2.

The presence of the unreported thujone enantiomers could not be detected in any of the 12 non-sage essential oils analyzed (EO1−EO12) or in several sage samples (EO13−EO20), as depicted in Table 1. With the large amounts of the reported stereoisomers in samples EO2, EO6, EO8, and EO12, even trace amounts of the unreported stereoisomers would be observable. With few samples available for analysis for oils, such as *Thuja occidentalis* (EO1 and EO2), *Achillea fragrantissima* (EO3), and *Tanacetum vulgare* (EO12), the sample size may impede the detection of the unreported enantiomers. The larger sample size of the *Artemisia vulgaris* (EO4−EO7) and *Artemisia herba-alba* (EO8−EO11) essential oils provides stronger support for the absence of (+)-α−thujone and (−)-β−thujone in those oils. In contrast, 72% of the sage essential oils analyzed (Table 2) contained the unreported enantiomers. A comparison of their relative concentrations of the thujone stereoisomers in samples A−U is displayed in Figure 6.

Unlike many monoterpene analytes that have relatively consistent enantiomeric ratios, the variation in the enantiomeric concentration of the unreported stereoisomers in the sage samples was dramatic. The enantiomeric excess (ee) for each sample was calculated and is displayed in Figure 7. The α enantiomers varied from 96% ee of the reported (−)-α enantiomer to 98% ee of the unreported (+)-α enantiomer.

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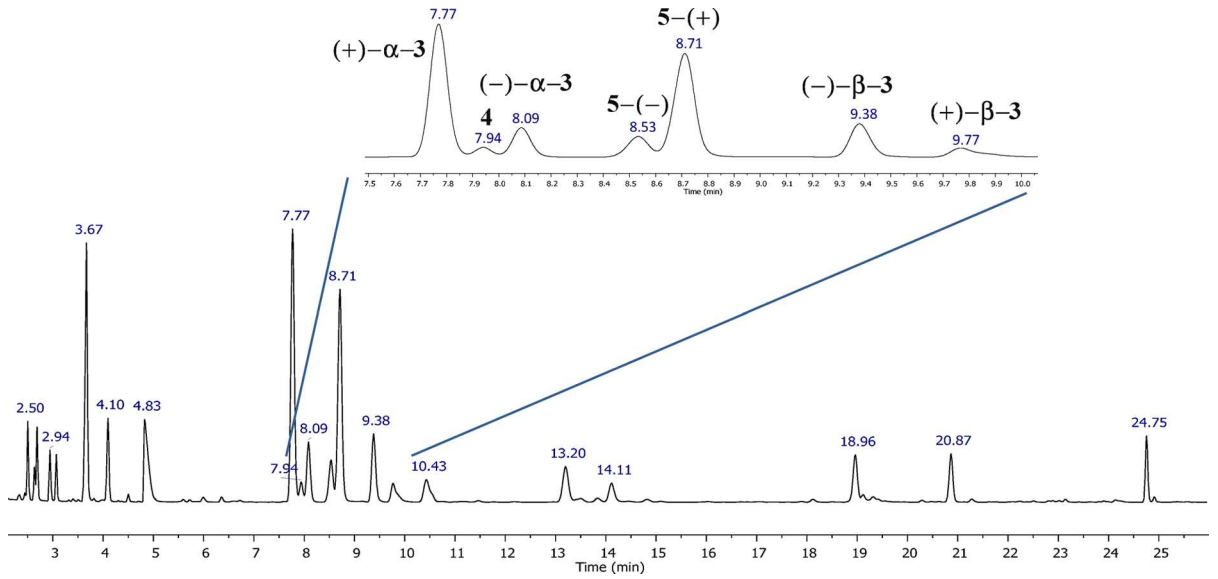

Figure 8. GC−MS total ion chromatograph of sample DS2.

Sample M contained the highest ratio (98% ee) of (+)-α/(−)-α at 16.2−0.2%, respectively. A total of 12 of the 21 samples containing the (+)-α enantiomer had this enantiomer as the major enantiomer, with ee ranging from 34 to 98%. Only 8 of the 21 samples had the reported (−)-α enantiomer as the major enantiomer, with the ee ranging from 28 to 96%. One sample (F) contains a racemic mixture of the α enantiomers. Interestingly, samples with the (+)-α enantiomer as the major enantiomer also possessed the unreported (−)-β enantiomer as the major enantiomer. The magnitude of the ee varied from the α enantiomers, with the ee for the β enantiomers being typically lower. A higher percentage of the samples possessed the (−)-β enantiomer (13 samples), while samples A and C contained the reported β enantiomer as the major enantiomer. Also, five samples were racemic with regard to the β enantiomers. Samples L and O possessed the highest ee (73%) of the unreported (−)-β to (+)-β enantiomer at a concentration of 3.8−0.6%.

The location of origin for most of the 41 samples was provided by the vendor (Supporting Information). On the basis of the provided information, there is no correlation between the country of origin and the presence of the unreported stereoisomers for the samples of sage essential oil analyzed. Additional data and analysis are required to determine the cause of the uctuating ee.

Because no unreported thujone isomers were detected in several samples, it is unlikely that the presence of the unreported stereoisomers occurred as artifacts of thermal treatment[33] in the production process. To confirm this, sample EO18, which contained no unreported stereoisomers, was heated at 165 °C for 18 h and then analyzed by GC−MS. While some epimerization between the diastereomers occurred, no unreported stereoisomers were detected, which corroborates that these are not formed by any post-thermal treatment of the essential oil. The possibility of unreported thujone stereoisomers forming during isolation of the sage essential oil is also unlikely because steam distillation of the plant parts was reported by all manufacturers as the method used to isolate the essential oils. Additionally, the essential oil obtained by hydrodistillation of commercially purchased dried sage plant (EO16) contained no unreported enantiomers by GC−MS.

Analysis of Thujone-Containing Teas, Spices, and Dietary Supplements. Because the essential oil of sage was found to possess varying amounts of the unreported stereoisomers, our efforts turned to analyzing consumable products, namely, dietary supplements, spices, and teas, as summarized in Table 3. The unreported stereoisomers could not be detected in the tea (T1−T6) or spice (S1−S6) samples analyzed, although the thujone concentration was found to vary considerably among the producers. Two of the dietary supplements (DS1 and DS2) were found to contain significant amounts of (+)-α- and (−)-β-thujone, such that they are the major enantiomers in both supplements. Both supplements also had similar concentrations of all four enantiomers and similar ee. Sample DS1 has a 62% ee of (+)-α enantiomer and a 42% ee in favor of (−)-β-thujone. Sample DS2 possessed the (+)-α enantiomer in a 63% ee and (−)-β-thujone in a 50% ee with the chromatogram displayed in Figure 8. Sample DS3 contained only the known enantiomers but in low concentration. Also noteworthy is the complete absence of thujone in samples DS4 and DS5. Sample DS4 contained camphor, accounting for 60% of the volatile components, and DS5 contained 71% furfural. While sage essential oils lacking thujone and containing significant amounts of camphor (DS5) have been reported,[34,35] those with significant amounts of furfural (DS4) have not been reported.

Using a synthetic sample of all four stereoisomers of thujone, (+)-α-thujone and (−)-β-thujone stereoisomers were detected for the first time. The sage essential oils containing the unreported stereoisomers varied greatly in their concentration from sample to sample. Some samples contained none of the unreported stereoisomers, while other contained only trace amounts of the reported stereoisomers. A rationale for the variability in enantiomeric composition is currently under investigation. Common dietary supplements were found to contain the unreported stereoisomers, prompting a need for an analysis of the biological activity of these compounds because thujone is a regulated substance. The stereoisomers were found in only 72% of the sage samples analyzed, suggesting the need for a larger sample base of non-sage thujone-containing essential oils. Additionally, the described concise synthesis and chiral resolution of thujone should enable a reassessment of

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other thujone-containing plants, especially in instances where their pharmacological properties are attributed to (−)-α- or (+)-β-thujone.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jafc.6b01065.

Vendor information for essential oils, vendor information for essential oils containg the unreported stereisomers, vendor information for consumable products of sage, and ^{1}H and ^{13}C NMR spectra of compounds **2** and **3** (PDF)

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS USED

HS-SPME, headspace solid-phase microextraction; GC−MS, gas chromatography−mass spectrometry; DMF, dimethylformamide; THF, tetrahydrofuran

■ REFERENCES

(1) Höld, K. M.; Sirisoma, N. S.; Ikeda, T.; Narahashi, T.; Casida, J. E. α-Thujone (the active component of absinthe): γ-Aminobutyric acid type A receptor modulation and metabolic detoxification. *Proc. Natl. Acad. Sci. U. S. A.* **2000**, *97*, 3826−3831.

(2) Lachenmeier, D. W.; Uebelacker, M. Risk assessment of thujone in foods and medicines containing sage and wormwood − evidence for a need of regulatory changes? *Regul. Toxicol. Pharmacol.* **2010**, *58*, 437−443.

(3) Pelkonen, O.; Abass, K.; Wiesner, J. Thujone and thujone-containing herbal medicinal and botanical products: Toxicological assessment. *Regul. Toxicol. Pharmacol.* **2013**, *65*, 100−107.

(4) Herraiz-Peñalver, D.; Ortiz de Elguea-Culebras, G.; Sánchez-Vioque, R.; Santana Méridas, O. Identification of a hybrid species of sage (*Salvia officinalis* L. x *S. lavandulifolia* subsp. *lavandulifolia*) through the study of the essential oil. *J. Essent. Oil Res.* **2015**, *27*, 363−372.

(5) Santos-Gomes, P. C.; Fernandes-Ferreira, M. Essential oils produced by in vitro shoots of sage (*Salvia officinalis* L.). *J. Agric. Food Chem.* **2003**, *51*, 2260−2266.

(6) Tsiri, D.; Graikou, K.; Poblocka-Olech, L.; Krauze-Baranowska, M.; Spyropoulos, C.; Chinou, I. Chemosystematic value of the essential oil composition of *Thuja* species cultivated in Poland—Antimicrobial activity. *Molecules* **2009**, *14*, 4707−4715.

(7) Perry, N. B.; Anderson, R. E.; Brennan, N. J.; Douglas, M. H.; Heaney, A. J.; McGimpsey, J. A.; Smallfield, B. M. Essential oils from dalmatian sage (*Salvia officinalis* L.): Variations among individuals, plant parts, seasons, and sites. *J. Agric. Food Chem.* **1999**, *47*, 2048−2054.

(8) Rohloff, J.; Mordal, R.; Dragland, S. Chemotypical variation of tansy (*Tanacetum vulgare* L.) from 40 different locations in Norway. *J. Agric. Food Chem.* **2004**, *52*, 1742−1748.

(9) Sivropoulou, A.; Nikolaou, C.; Papanikolaou, E.; Kokkini, S.; Lanaras, T.; Arsenakis, M. Antimicrobial, cytotoxic, and antiviral activities of *Salvia fructicosa* essential oil. *J. Agric. Food Chem.* **1997**, *45*, 3197−3201.

(10) Santos-Gomes, P. C.; Fernandes-Ferreira, M. Organ- and season-dependent variation in the essential oil composition of *Salvia officinalis* L. cultivated at two different sites. *J. Agric. Food Chem.* **2001**, *49*, 2908−2916.

(11) Umano, K.; Hagi, Y.; Nakahara, K.; Shoji, A.; Shibamoto, T. Volatile chemicals identified in extracts from leaves of Japanese mugwort (*Artemisia princeps pamp.*). *J. Agric. Food Chem.* **2000**, *48*, 3463−3469.

(12) Waidyanatha, S.; Johnson, J. D.; Hong, S. P.; Robinson, V. G.; Gibbs, S.; Graves, S. W.; Hooth, M. J.; Smith, C. S. Toxicokinetics of α-thujone following intravenous and gavage administration of α-thujone or α- and β-thujone mixture in male and female F344/N rats and B6C3F1 mice. *Toxicol. Appl. Pharmacol.* **2013**, *271*, 216−228.

(13) Rice, K. C.; Wilson, R. S. (−)-3-Isothujone, a small nonnitrogenous molecule with antinociceptive activity in mice. *J. Med. Chem.* **1976**, *19*, 1054−1057.

(14) Foster, A. J.; Hall, D. E.; Mortimer, L.; Abercromby, S.; Gries, R.; Gries, G.; Bohlmann, J.; Russell, J.; Mattsson, J. Identification of genes in *Thuja plicata* foliar terpenoid defenses. *Plant Physiol.* **2013**, *161*, 1993−2004.

(15) Croteau, R. Biosynthesis and catabolism of monoterpenoids. *Chem. Rev.* **1987**, *87*, 929−954.

(16) Verzera, A.; la Rosa, G.; Zappalà, M.; Cotroneo, A. Essential oil composition of different cultivars of bergamot grown in Sicily. *Ital. J. Food Sci.* **2000**, *12*, 493−501.

(17) Frizzo, C. D.; Lorenzo, D.; Dellacassa, E. Composition and seasonal variation of the essential oils from two mandarin cultivars of southern Brazil. *J. Agric. Food Chem.* **2004**, *52*, 3036−3041.

(18) Williams, J. D.; Campbell, M. A.; Jaskolka, M. C.; Xie, T. *Artemisia vulgaris* L. chemotypes. *Am. J. Plant Sci.* **2013**, *04*, 1265−1269.

(19) Mondello, L.; Costa, R.; Sciarrone, D.; Dugo, G. The chiral components of citrus oils. In *Citrus Oils Composition, Advanced Analytical Techniques, Contaminants, and Biological Activity. Medicinal and Aromatic Plants—Industrial Pro les*; CRC Press: Boca Raton, FL, **2011**; Vol. *49*, pp 349−403.

(20) Wajs, A.; Urbańska, J.; Zaleśkiewicz, E.; Bonikowski, R. Composition of essential oil from seeds and cones of *Abies alba*. *Nat. Prod. Commun.* **2010**, *5*, 1291−1294.

(21) König, W. A.; Rieck, A.; Hardt, I.; Gehrcke, B.; Kubeczka, K.-H.; Muhle, H. Enantiomeric composition of the chiral constituents of essential oils. Part 2: Sesquiterpene hydrocarbons. *J. High Resolut. Chromatogr.* **1994**, *17*, 315−320.

(22) Do, T. K. T.; Hadji-Minaglou, F.; Antoniotti, S.; Fernandez, X. Authenticity of essential oils. *TrAC, Trends Anal. Chem.* **2015**, *66*, 146−157.

(23) Mosandl, A.; Braunsdorf, R.; Bruche, G.; Dietrich, A.; Hener, U.; Karl, V.; Köpke, T.; Kreis, P.; Lehmann, D.; Maas, B. New methods to assess authenticity of natural avors and essential oils. In *Fruit Flavors*; Rouseff, R. L., Leahy, M. M., Eds.; American Chemical Society (ACS): Washington, D.C., 1995; ACS Symposium Series, Vol. *596*, Chapter 9, pp 94−112, DOI: 10.1021/bk-1995-0596.ch009.

(24) Tabacchi, R.; Saturnin, G. C. *A Guide to the Analysis of Chiral Compounds by GC*; Restek Corporation: Bellefonte, PA, 1997; http://www.restek.com/pdfs/59889.pdf (accessed Jan 11, 2016).

(25) Walch, S. G.; Kuballa, T.; Stühlinger, W.; Lachenmeier, D. W. Determination of the biologically active flavor substances thujone and camphor in foods and medicines containing sage (*Salvia officinalis* L.). *Chem. Cent. J.* **2011**, *5*, 44.

(26) Máthé, I.; Olah, L.; Máthé, Á.; Miklossy, V. V.; Bernáth, J.; Blunden, G.; Patel, A. V.; Máthé, I. Changes in the essential oil

production of *Salvia officinalis* under climatic conditions of the temperate belt. *Planta Med.* **1992**, *58*, 680.
(27) Lakusić, B. S.; Ristić, S. M.; Slavkovska, V. N.; Stojanović, D. L.; Lakusić, D. V. Variations in essential oil yields and compositions of *Salvia officinalis* at different developmental stages. *Bot. Serb.* **2013**, *37*, 127−138.
(28) Estévez, M.; Ventanas, S.; Ramirez, R.; Cava, R. Analysis of volatiles in porcine liver pátés with added sage and rosemary essential oils by using SPME-GC-MS. *J. Agric. Food Chem.* **2004**, *52*, 5168−5174.
(29) Journet, M.; Cai, D.; DiMichele, L. M.; Larsen, R. D. Highly efficient synthesis of α,β-acetylenic aldehydes from terminal alkynes using DMF as the formylating reagent. *Tetrahedron Lett.* **1998**, *39*, 6427−6428.
(30) French, L. G. Isolation and structure elucidation of the terpene β-thujone from cedar leaf oil. *J. Chem. Educ.* **2011**, *88*, 829−831.
(31) Oppolzer, W.; Pimm, A.; Stammen, B.; Hume, W. Palladium-catalyzed intramolecular cyclizations of olefinic propargylic carbonates and application to the diastereoselective synthesis of enantiomerically pure (−)-α-thujone. *Helv. Chim. Acta* **1997**, *80*, 623−639.
(32) Mamane, V.; Gress, T.; Krause, H.; Fürstner, A. Platinum- and gold-catalyzed cycloisomerization reactions of hydroxylated enynes. *J. Am. Chem. Soc.* **2004**, *126*, 8654−8655.
(33) Stolle, A.; Ondruschka, B.; Hopf, H. Thermal rearrangements of monoterpenes and monoterpenoids. *Helv. Chim. Acta* **2009**, *92*, 1673−1719.
(34) Tucker, A. O.; Maciarello, M. J. Essential oils of cultivars of dalmatian sage. *J. Essent. Oil Res.* **1990**, *2*, 139−144.
(35) De Martino, L.; Roscigno, G.; Mancini, E.; De Falco, E.; De Feo, V. Chemical composition and antigerminative activity of the essential oils from five *Salvia* species. *Molecules* **2010**, *15*, 735−746.

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