Isolation of (*R*)-(+)-Pulegone from the European Pennyroyal Mint, Mentha Pulegium

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Abstract: Students obtain, via steam distillation, pennyroyal essential oil that is analyzed by capillary gas chromatography–mass spectrometry. TLC experiments establish conditions for preparative scale purification of the major oil component via flash column chromatography. The terpene obtained, (*R*)-(+)-pulegone, is characterized spectroscopically, employing at a minimum mass spectral molecular ion and fragmentation patterns, IR, and 300-MHz ^{1}H NMR. Optionally, ^{13}C and 2-D correlation NMR spectra can be utilized to enable unambiguous assignment of all C and H resonances. The project has been successfully incorporated into our upper-level advanced organic chemistry laboratory. The experiment provides opportunities for instruction in and experience with a wide variety of chromatographic and spectroscopic methods. Further, it centers on a plant and natural product well suited for the discussion of contemporary health care issues surrounding nontraditional/alternative medicine and herbal remedies.

Introduction

Projects involving the isolation and structure elucidation of natural products continue to fascinate students. Long-time standards include isolation of the terpenes, limonene, carvone, eugenol, and trimyristin and the alkaloid, caffeine. Most all currently utilized laboratory texts incorporate one or two of these exercises. There have been relatively few additions to this menu over the past two decades [1]. One difficulty comes in identifying readily available plant sources that provide an extract sufficiently rich in one component such that separation and purification does not pose too daunting a challenge for the undergraduate student.

A survey of the literature of commercially significant essential oils revealed that *Mentha pulegium*, a member of the mint family, produces an essential oil comprised largely (80 to 90%) of the monoterpene, (*R*)-(+)-pulegone **(1)** [2]. That the material actually possesses pleasant olfactory properties and intriguing folk-medical connections provided further impetus for the development of this project.

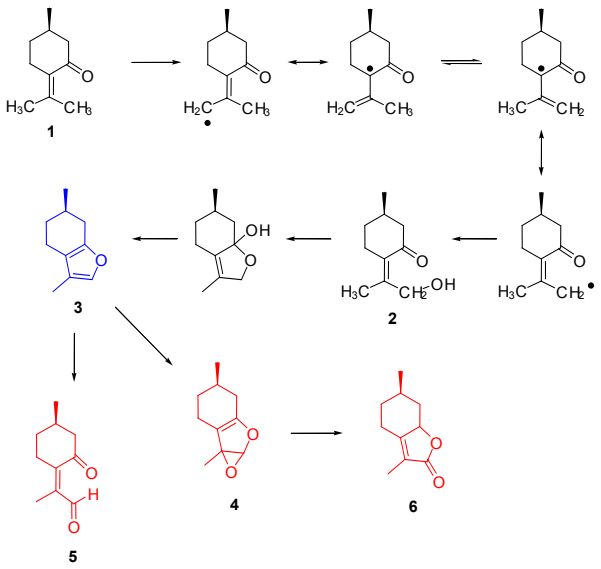
Botanical Considerations

The pennyroyal plant is classified in the family *Labitatae*. It occurs widely across the majority of Europe and parts of Asia. Also referred to as pulegium, run-by-the-ground, lurk-in-the-ditch, pudding grass, and piliolerial, it is distinct from American pennyroyal, *Hedeoma pulegioides*, another pulegone producing mint. A variety of etymological pedigrees have been suggested. Pliny dubbed the plant pulegium in ancient Rome where it had come into use as a flea repellent (L. pulex = flea) [3]. Morphologically the plant is a low growing, prostrate spreading herb with leaves that range in shape from ovate to orbicular. It produces lilac-colored flowers. It is cultivated in Europe and grows particularly well in fertile, moist soils in Mediterranean coastal regions. The oil has been used in flavor and fragrance applications and the derived pulegone employed for the commercial synthesis of menthol.

Folk Medicine—Natural is not Always Better

Interestingly, a search for pennyroyal on the Web will retrieve many more sites devoted to the ersatz Seattle-based grunge band Nirvana and its late lead singer Kurt Cobain than those with chemical or botanical content. It turns out that Cobain penned a tune, “Pennyroyal Tea,” which appeared on an album entitled (ironically?) “in Utero”. Cobain writes—“Sit and drink Pennyroyal Tea; Distill the life that’s inside of me.” In fact, pennyroyal extracts have been touted since antiquity as offering a means of inducing abortion. [4] A scientific basis for the effectiveness of this treatment has not been established. If pulegone, or one of the minor oil constituents, does have the ability to terminate pregnancy, it is an agent with very low therapeutic index and its effect is likely attributable to generalized systemic toxicity rather than selective stimulation of uterine tissue. The 16th-century English herbalist Gerard recommends, “Pennie Royall boiled in wine and drunken, provoketh the monthly terms, bringeth forth the secondine, the deade childe and unnatural birth” [5]. Herbalist pharmacopoeias throughout the 18th and 19th centuries cite the plant for use as a carminative, diaphoretic, emmenagogue, and sedative. Adding to its panacea status are claims for antispasmodic and anti-infective capabilities as well as utility for treating gout and colic.

Americans are turning in ever-greater numbers to practitioners of alternate medicine and to “natural” therapeutics [6]. A part of this trend involves renewed interest in herbal products purchased at health food stores and large chain pharmacy retailers and utilized as supplements, preventatives, and curatives. A surprisingly large segment of the populace is willing to self-treat with these botanicals although they are marketed without FDA oversight and are exempt from regulations stipulating proof of efficacy and safety. Many of these same individuals remain highly skeptical of new chemical entities introduced by the pharmaceutical giants as the culminations of 15-year, 800-million-dollar research and development efforts. [7] This is despite the fact



Scheme 1. Pulegone Metabolism

that pharmaceutical development sees the lion's share of investment made in conducting controlled, large-scale clinical trials required by the FDA to demonstrate safety and effectiveness. An examination of the use of pennyroyal in folk medicine offers an opportunity to discuss herbal medicine in the light of its potential for harm as well as good.

The potentially devastating consequences of pulegone ingestion are documented in cold clinical terms by Anderson et al. [8]. In their Annals of Internal Medicine paper the authors summarize experiences with recent cases of acute pennyroyal poisoning and provide a retrospective of the medical literature identifying previous such incidents. They uncover a total of five fatalities associated with multiple organ failure directly attributable to the consumption of pennyroyal derived teas or oils. One of Anderson's case studies was a 24-year-old California woman. Her tragic death, brought on by a fatal combination of untreated ectopic pregnancy and self-medication with pennyroyal, is chronicled in deeply personal terms by Santa Clara Valley Metro reporter Gordon Young in his piece, "Lifestyle on Trial" [9]. The story also captures some of the tensions at work between traditional medical practice and homeopathy. There are also reports in the medical literature of infant poisonings stemming from ingestion of home-brewed mint teas [10]. Such brews, known as "yerba buena," have been a part of traditional Hispanic practice for comforting infants with digestive difficulties and colic. A variety of additional native mint species, including those in the genus Pycanthemum (mountain mints), afford oils characterized by high pulegone content. This is important to note as many of the warnings, now fairly widely understood regarding the consumption of pennyroyal containing teas and elixirs, have not always transferred to these species. Infants and young children are especially susceptible to the hepato- and nephrotoxicities associated with pennyroyal and its metabolic products.

Pulegone Metabolism and Toxicity

Pulegone is acted upon by specific cytochrome P-450 oxidase isoforms in a metabolic pathway that culminates in the production of highly electrophilic metabolites. These toxins proceed to alkylate tissue proteins leading to cell death. Liver, kidney tubules, bronchial alveoli, and cerebral tissues suffer particularly devastating pathological hits. Studies on pulegone metabolism have been reviewed [11] and are summarized in Scheme 1.

Hydrogen atom abstraction from the anti primary allylic carbon in pulegone **1** generates a conjugated radical. (*E*)/(*Z*) isomerization followed by radical hydroxylation involving the heme cofactor affords an allylic alcohol **2** poised for cyclization and aromatization via dehydration. The resulting menthofuran **3** is the proximate electrophile. Subsequent P-450 mediated oxidation can afford both an epoxide **4** and γ-ketoenal **5**. Topoisomerization of either of these species generates mintfuranone **6**. Species **4–6** are ultimate electrophiles capable of forming protein adducts. These species, as well as pulegone itself, also form glutathione conjugates in the normal course of xenobiotic detoxification. The disposition of pulegone and its mechanism of liver injury are quite analogous to that of acetaminophen, as is the particular sensitivity of infants and young children in whom glutathione stores can be quickly depleted leading to build up of toxic metabolites.

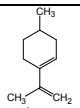
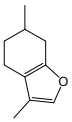
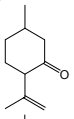
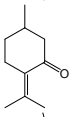
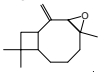
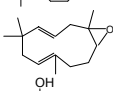
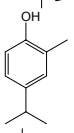
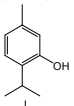
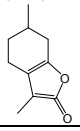
So, should one sit and drink pennyroyal tea? Consumption of large amounts of the neat essential oil have been inextricably linked to fatalities. Infants and young children should definitely not consume any. The small quantities of pulegone present in steeped teas have not yet been shown to pose a severe health risk in adults with normal liver function; however, the proven hepatotoxicity of pulegone and its metabolites should mitigate against consumption of pulegone containing brews. This is especially the case for those taking other xenobiotics (drugs or herbal products), which either induce P-450 enzymes or require them for detoxification.

Results and Discussion

Starwest Botanicals Inc. supplies *Mentha pulegium* of Spanish origin which we obtain at a local health food emporium [12]. The retail price is currently about $14 per pound. Students are not informed of the identity of the plant species until after they have identified the major component of the essential oil. Steam distillation of the dried plant material typically affords about 1% by weight of essential oil rich (60–90%) in pulegone content. The quality and composition of steam-distilled oils is a function of temperature and degree of contact between oil components and hot water. Artifacts, particularly oxidized and hydrolyzed compounds, should be expected. Here, good results are obtained by floating the dry plant material atop the water in the distillation flask. The distillate mixture is extracted into methylene chloride or ether, dried, and rotary evaporated to afford crude oil.

The crude oil is examined by capillary GC–MS to gauge the number of components and percentage composition. The major component is noted and its mass spectrum obtained. Eventually, students can return to the total ion chromatogram generated and utilize a matching algorithm within the NIST mass spectral database [13] to verify their structural assignment and attempt to identify minor constituents. A typical total-ion chromatogram is provided in Figure 1 (details in experimental section), and selected components are listed in Table 1 [14]. In addition to pulegone (RT = 14.76 min), a number of other, minor components give very good to excellent NIST database matches. (Table 1).

Table 1. Selected Components from the Total-Ion Chromatogram

Compound	RT	% of total	SI match index	RSI match index	Name
	4.93	0.83	905	906	*p*-mentha-3,8-diene
	5.85	0.39	905	926	3-methylcyclo-hexanone
	9.43	0.74	825	838	menthofuran
	11.56	1.89	916	921	isopulegone
	13.10	60.1	932	938	pulegone
	19.20	3.67	866	872	verbenone
	20.36	0.62	913	914	caryophyllene oxide
	21.55	1.13	865	887	CA 19888-34-7
	24.74	0.24	891	896	carvacrol
	25.27	0.33	906	908	*m*-thymol
	26.86	3.25	914	918	mint furanone

After reading Still's paper [15], TLC is conducted in a series of solvents in order to identify viable conditions for preparative scale flash column chromatographic isolation of the major component. Students compare a series of developing solvents comprised of various mixtures of hexane and ethyl acetate. Visualization is done using ethanolic vanillin/H_2SO_4. We find that a solvent mixture of 5% ethyl acetate in hexane provides good separation for the flash column separation and yields pulegone of sufficient purity (>90%) for spectroscopic analysis.

Spectroscopic Analysis

IR Spectroscopy. IR spectroscopy (Figure 2) was conducted on a Mattson Polaris FTIR with the neat liquid. Diagnostic peaks are present at 1678 cm^{-1} for the conjugated carbonyl and 1616 cm^{-1} for the alkene C=C stretching.

NMR Spectroscopy. ^1H NMR spectroscopy (Figure 3) was carried out on JEOL Eclipse 300-MHz spectrometer. Even though unambiguous assignment of all the resonances is challenging, this information in conjuction with the mass spectral data and IR spectrum is sufficient to correctly identify the unknown [16].

Utilization of ^{13}C NMR data (Figure 4), ^1H-^1H correlations from DQF-COSY (Figure 5) and the inverse detected proton–carbon correlation technique, HMQC (Figure 6) renders complete assignment of all C and H resonances manageable. Pulegone is an exemplary molecule for providing students with an introduction to 2-D correlation spectroscopy.

The sp^2 carbons are easily assigned based on interpretation of chemical shifts. The aliphatic carbons, with the exception of the alpha carbon, C_7, present more of a challenge; however, as can be seen below, the ^1H–^{13}C correlations observed in the HMQC spectrum allow resolution of these issues.The DQF-COSY spectrum (Figure 5) unveils the proton–proton coupling interactions. A good interpretive entry point is with the most highly deshielded H_j, the pseudoequatorial 2^o allylic proton. (A small anisotropic effect from the gauche C–C ring bond is known to result in equatorial protons resonating at slightly higher frequency than their axial counterparts.) A strong off-diagonal signal identifies the geminally interacting H_h and the two vicinally coupled, 2^o protons H_d (pseudoequatorial so further downfield) and H_b are easily discerned. Alternately, starting with the obvious up-field doublet for the methyl group leads to identification of methine f. Very weak correlations for f with b and d (not labeled) can be found. This leaves two diastereotopic methylene protons, i and g, which are α to the carbonyl that become assignable by the process of elimination. Primary allylic methyl groups c and e give rise to singlets at 1.73 and 1.94 ppm, respectively. In acyclic systems one would expect the methyl group cis to the carbonyl to be furthest downfield; however, in pulegone the conjugated carbonyl is locked into a cisoid conformation.

The HMQC spectrum (Figure 6) yields ^1H–^{13}C correlations. Conspicuous are the sets of diastereotopic H pairs (H_j, H_h), (H_i, H_g), and (H_d, H_b) which spin label and identify C_4, C_7, and C_6, respectively. Hydrogen correlations also aid in the assignment of the methyl groups.

Molecular Modeling Component

The project can be augmented with a simple molecular modeling exercise. Computational work was performed in our case using HyperChem (Ver. 5), although any software capable of simple semiempirically based geometry optimization routines could be employed. Using HyperChem, the structure of (*R*)-(+)-pulegone was built. A geometry optimization was conducted using PM3 and yielded the structure shown in Figure 7 and the data in Table 2 [17].

Relevant dihedral angles were determined and the Karplus curve consulted in order to predict some of the easily observable first-order coupling constants, specifically for vicinal couplings of protons j and i. The clean doublet of triplets for the most deshielded proton, the pseudoequatorial 2^o allylic H_j, is then easily understood in terms of equivalent coupling to pseudoequatorial H_d and pseudoaxial H_b.

Experimental Section

Plant material, (100 g) of dry pennyroyal leaves and stems were floated on top of water (approximately 1000 mL) in a 2-L round-bottom flask equipped with a distillation head connected to a condenser. Heating was conducted with a mantle and distillate collected until it ran clear and without minty odor. The aqueous

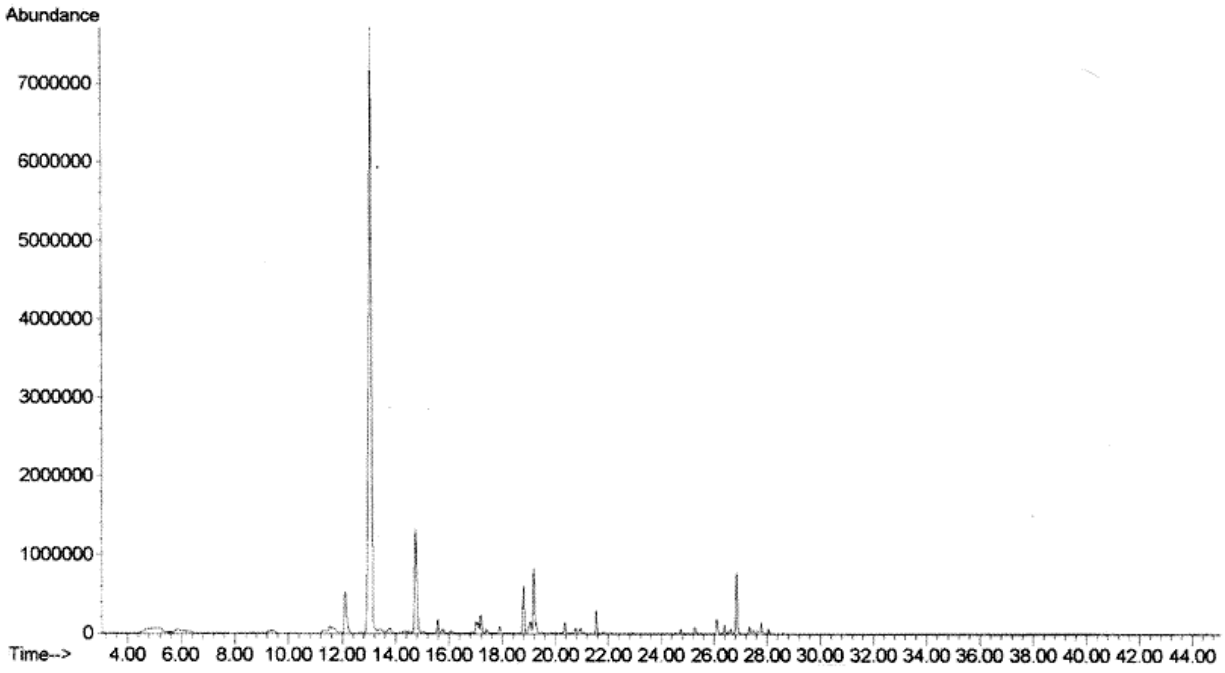


Figure 1. Total-ion chromatogram of crude pennyroyal oil.

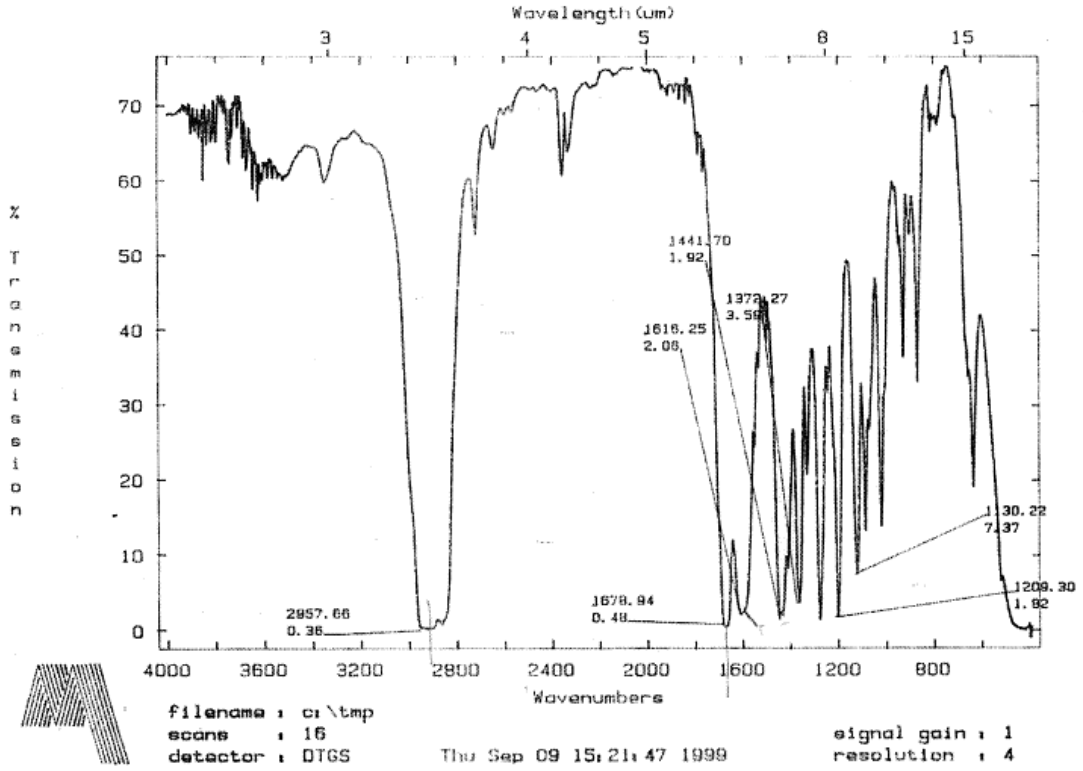


Figure 2. Infrared spectrum of pulegone (neat).

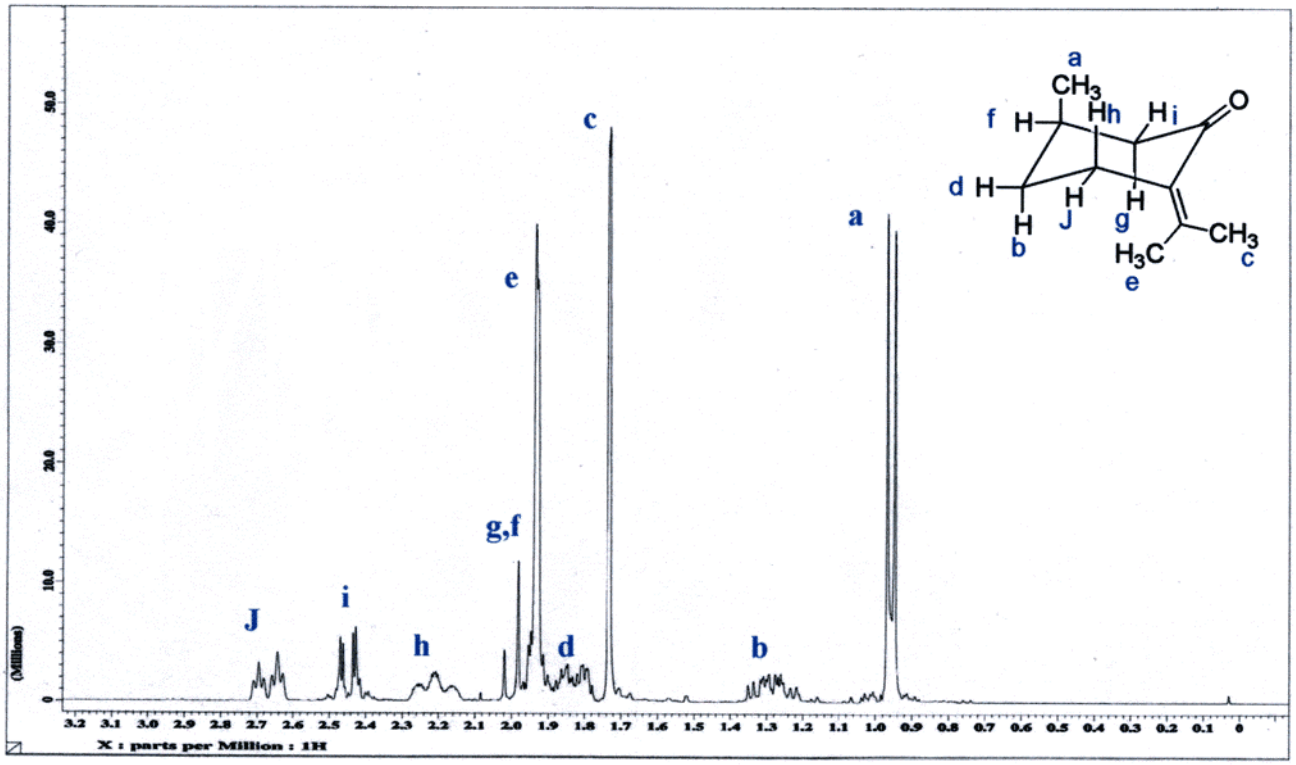


Figure 3. Pulegone ^{1}H NMR ($CDCl_3$) with assignments.

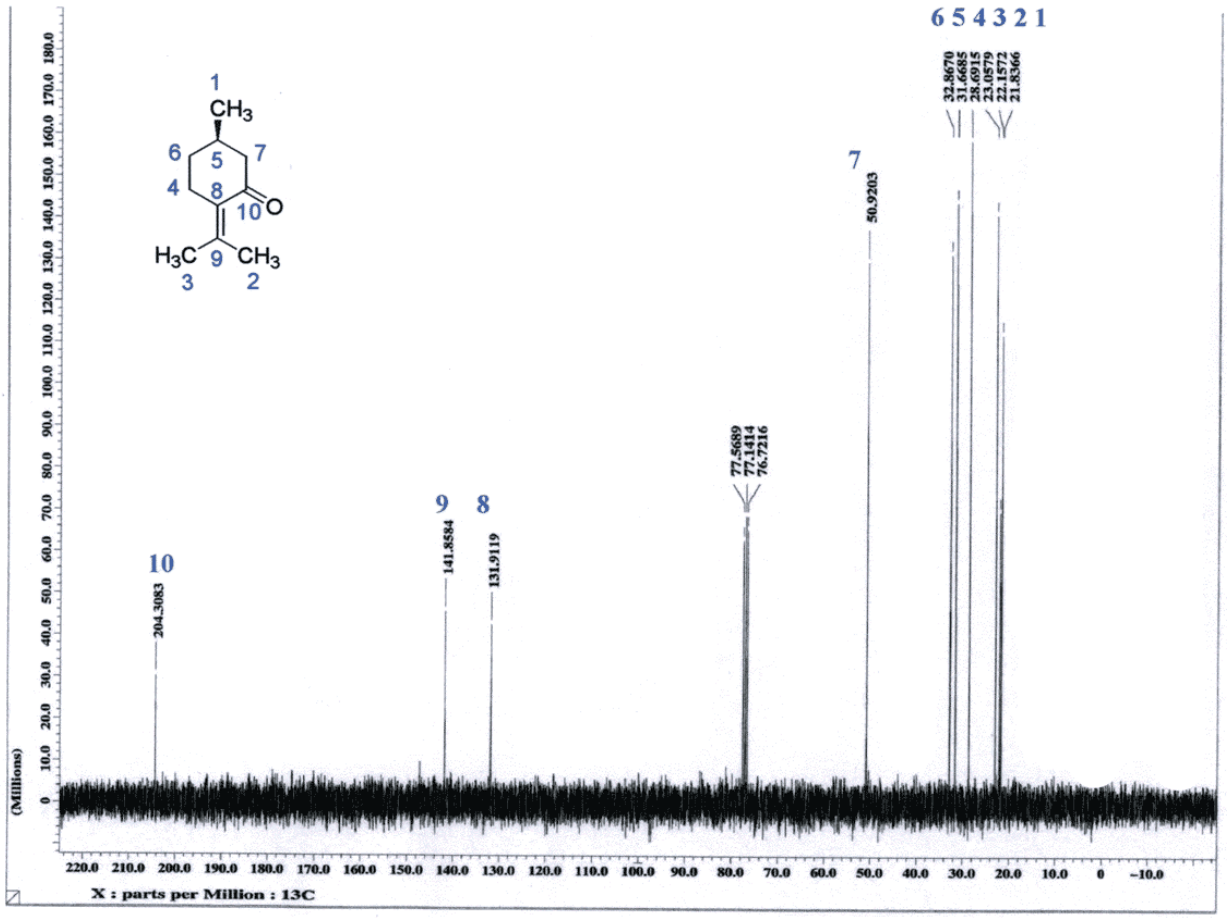


Figure 4. Pulegone ^{13}C NMR with assignments.

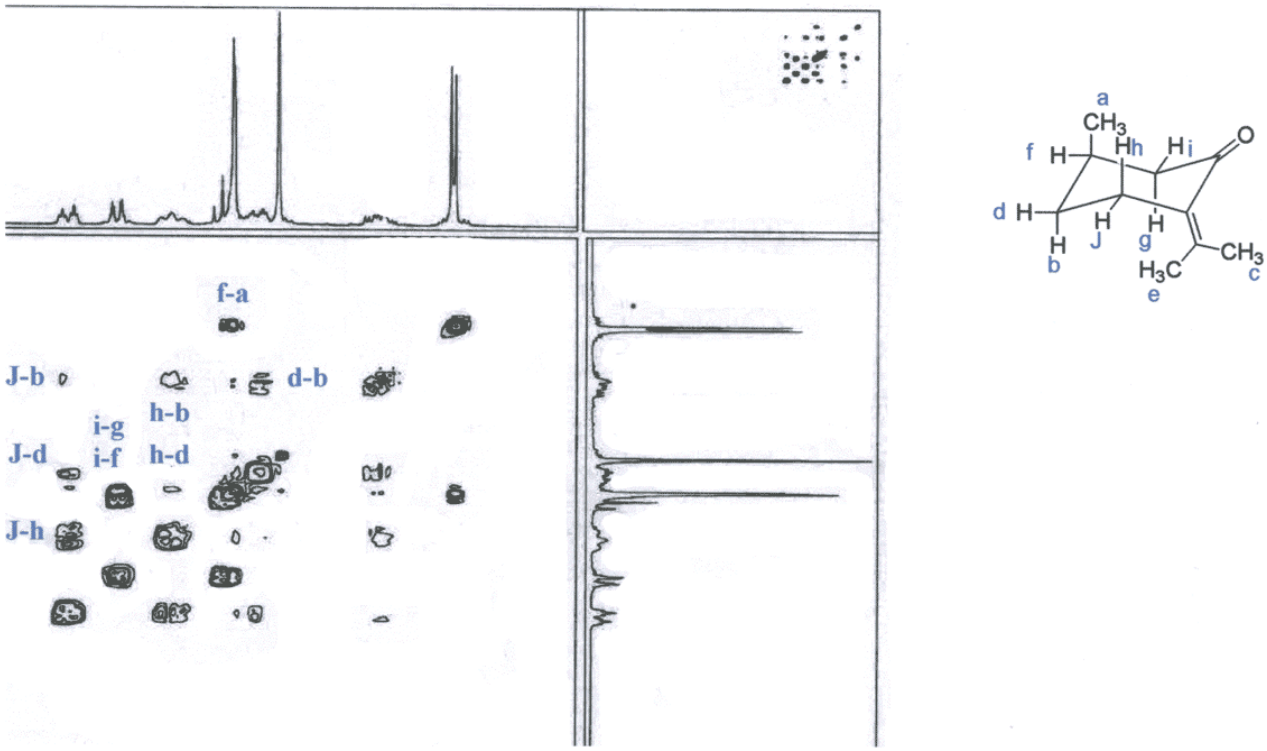


Figure 5. Pulegone DQF-COSY spectrum with off diagonal correlations.

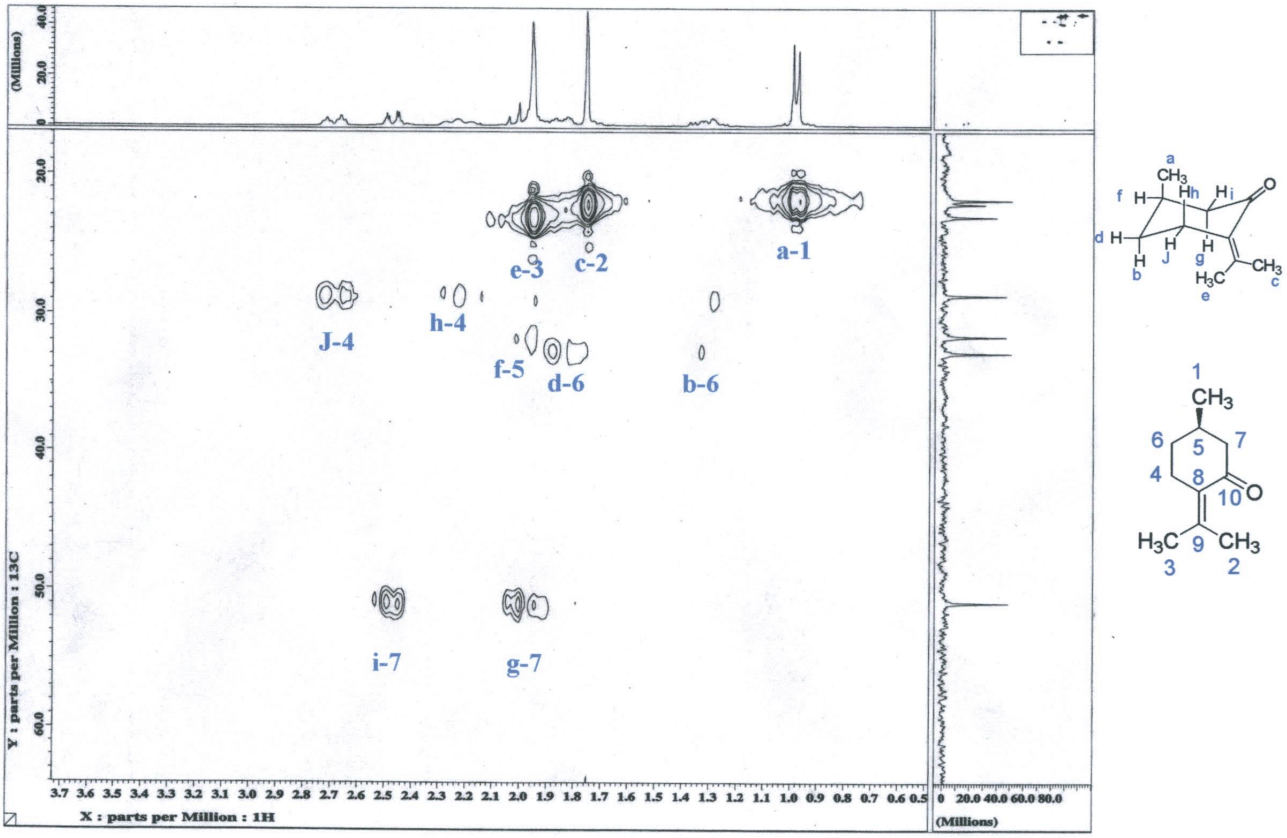


Figure 6. Pulegone HMQC inverse detected heteronuclear correlations.

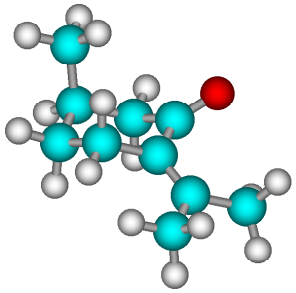


Figure 7. Structure of (*R*)-(+)-pulegone obtained from geometry optimization using the semiempirical PM3 method.

Table 2. Structural Properties of (*R*)-(+)-Pulegone

Protons	Dihedral Angle (PM3 Calculated)	*J* (Karplus Predicted)	*J* (Observed)
dh	56.4°		
dj	59.7°	3 Hz	4.1 Hz
bh	172°		
bj	56.0°	3 Hz	4.1 Hz
df	66.8°		
bf	48.8°		
fi	67.0°	1 Hz	2.0 Hz
fg	48.8°		

mixture was then extracted with ether (3 × 100 mL). The combined ether layers were dried over sodium sulfate and solvent removed by rotary evaporation to return crude oil (approximately 1% by dry weight). A sample of the essential oil was prepared for GC–MS analysis by dissolving about 0.005g in 1 mL of ether. GC–MS was carried out on an HP5890 Series II/5971 instrument on an HP-Innowax column (30 m × 0.25 mm × 0.25 µm). Helium flow rate was 1 ml/min. The injection port temperature was set at 250 °C. A temperature gradient run was conducted beginning at 80 °C for 3.0 min followed by an increase of 5°/min to a maximum of 240 °C and held for 10 min. The mass spectrometer interface was at 280 °C and masses between 30 and 550 were scanned. A typical total ion chromatogram appears in Figure 1 with the major component, pulegone, eluting at 13.03 min. The crude product (0.8–1.2 g) was subjected to flash column chromatographic separation on silica gel (Merck, grade 60) eluting with 5% ethyl acetate/hexanes. Fractions (25 × 50 mL) were collected and those containing pulegone (R_f = 0.2 on silica gel plates with 5% ethylacetate/hexanes) were combined, dried, and rotary evaporated to give pulegone (0.5–0.8 g) in greater than 90% purity.

IR (neat) 2957, 1679, 1616, 1441, 1372 1209, 1130 cm^{-1}; ^1H NMR ($CDCl_3$, δ) 2.68 (d of t, H_j); 2.45 (d of d, H_i); 2.20–1.90 (m, H_g, H_f); 1.90 (s, H_e);1.85 (m, H_d); 1.74 (s, H_c); 1.28 (m, H_b); 0.95 (d, H_a); ^{13}C NMR ($CDCl_3$, δ) 204.31, 141.86, 131.91, 50.92, 32.87, 31.67, 28.69, 23.06, 22.16, 21.84; LRMS (70 eV), m/e (relative intensity) 152 (M^+, 47), 137 (21), 109 (47), 82 (33), 81 (100), 67 (75), 41 (38), 39 (35).

Supporting Materials. A student handout is available as supporting material (http://dx.doi.org/10.1007/s008970205991b)

References and Notes

1. Some notable examples include (a) curcumin from turmeric: Anderson, A. M.; Mitchell, M. S.; Mohan, R. S. *J. Chem. Educ.* **2000,** *77,* 359–360; (b) artemisin and related sesquiterpenes from *Artemisinia annua*: Roth, R. J.; Acton, N. *J. Chem. Educ.* **1989,** *66,* 349–350; (c) piperine from black pepper: Epstein, W. W.; Netz, D. F.; Seidel, J. L. *J. Chem. Educ.*, **1993,** *70,* 598–599; (d) thiarubrine from *Abrosia artemissiifolia*: Reyes, J.; Morton, M.; Downum, K.; O'Shea, K. E. *J. Chem. Educ.* **2001,** *78,* 781–783.
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13. NIST '98 Mass Spectral Library with Windows Search Program (Version 1.6), The NIST Mass Spectrometry Data Center, U.S. Department of Commerce, National Institute of Standards and Technology, Gaithersburg, MD, 1998.
14. Two indices quantifying the similarity of the match are given. The SI index considers all peaks in the sample in making a comparison with library spectra; the RSI index ignores masses present in the sample that do not appear in the reference spectrum. 1000 is a perfect score. Matches over 900 are considered excellent and over 800 very good.
15. Still, W. C.; Kahn, M.; Mitra, A. *J. Org. Chem.* **1978,** *43,* 2923–2926.
16. Students are instructed to first work with the mass spectral data and to use the abundance of the M + 1 peak to enable determination of the molecular formula as $C_{10}H_{16}O$. An index of hydrogen deficiency calculation unveils a total of three degrees of unsaturation. The low-frequency carbonyl stretch is consistent with α,β-unsaturation. Two methyl singlets in the 1.7–2.0 ppm range and the lack of any vinyl hydrogens enables construction of one intact isoprene unit (Carbons 2, 3, 8, 9, 10). With this substructure in hand, one site of unsaturation is left to account for. The presence of an aliphatic methyl group (doublet) is indicated and chemical shifts for the as yet unaccounted for protons suggest methylene groups at an allylic position and α-to the carbonyl. At this point it is apparent that the molecule incorporates a six-membered ring, the final unsaturation. There remain only two reasonable means of completing the molecule which differ as to whether the methyl group is appended to C-5 or C-6. Recourse to either interpretation of the mass spectral fragmentation pattern, or speculation based on the isoprene rule (students are informed of the fact that the structure is a terpene) will allow this

final element of connectivity to be deduced. This line of reasoning will allow for a final structure determination in the absence of any 2-D or even ^{13}C NMR data.

17. 17. HyperChem 5.02, Hypercube Inc., Gainesville, FL. The semi-empirical PM3 method was applied. The convergence limit equals 0.01 and the iteration limit equals 50 with the Polak-Ribiere conjugate gradient employed until a RMS gradient of 0.0 kcal ang^{-1} mol^{-1} was achieved.