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Isolation and Identification of Terpene Chlorohydrins Found in Cold-Pressed Orange Oil

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Three terpene chlorohydrins found in cold-pressed orange oil were concentrated by silica adsorption chromatography and purified by preparative HPLC. Formation of these chlorohydrins was determined to be the result of a reaction of *d*-limonene, the major component of cold-pressed oil, with hypochlorous acid, found in chlorinated treatment water used in the oil recovery process. NMR analyses indicated that the major chlorohydrin present was the diequatorially substituted (1*R*,2*R*,4*R*)-2-chloro-8-*p*-menthen-1-ol (**1**). The other two compounds were the diaxial trans stereoisomer, (1*S*,2*S*,4*R*)-2-chloro-8-*p*-menthen-1-ol (**2**), and the dichlorohydrin, (1*R*,2*R*,4*R*)-2,9-dichloro-8-*p*-menthen-1-ol (**3**).

KEYWORDS: Chlorohydrins; terpenes; *d*-limonene; citrus oil; hypochlorous acid

INTRODUCTION

Disinfection byproducts occurring in chlorine-treated water have been studied (*1*) and may have important implications in the process of manufacturing citrus essential oils. Preliminary screenings in the authors' laboratory of some commercial cold-pressed citrus oil samples by gas chromatography with a halogen detector revealed the presence of chlorinated compounds. Widespread chlorine sanitation in citrus processing led to the hypothesis of a reaction between hypochlorous acid (HOCl) and *d*-limonene, which is present at ∼90−95% in citrus oils. Potential sources of HOCl as a reactant include chlorinated treatment water used in the oil recovery process and sanitizers used in postharvest handling and process equipment cleaning.

The electrophilic addition of HOCl to alkenes is an established reaction mechanism for chlorohydrin formation. The reaction follows the Markovnikov rule with the hydroxyl group adding to the more substituted carbon. Reactions of HOCl with a variety of terpenes similar in structure to *d*-limonene, including limonene monoxide (*2*), α-pinene (*3*), and α-terpineol (*4*), have been reported. The formation of chlorinated monoterpenes during kraft pulp bleaching has been studied, and a multitude of reaction products have been identified (*5*).

Of particular importance to the present study, two major products of the aqueous chlorination of α-terpineol have been identified as diaxial and diequatorial *trans*-chlorohydrins (*4*). The diaxial product was observed only at acidic pH, readily losing HCl at basic pH to form the corresponding epoxide. NMR analyses of chlorohydrins formed by reaction with related terpenes have been reported (*6*−*9*).

The objectives of this study were to identify the three main chlorohydrin compounds detected in citrus essential oils and test a hypothesis regarding formation of these compounds from limonene and HOCl.

MATERIALS AND METHODS

Detection of Chlorohydrin Compounds. Commercial citrus oil samples were analyzed on both nonpolar and polar columns utilizing a Hewlett-Packard 5890 series II gas chromatograph (Avondale, PA) with an O-I Analytical model 5220 electrolytic conductivity detector (GC-ELCD) (College Station, TX). Nonpolar column conditions were as follows: 30 m × 0.53 mm i.d., 1.5 μm DB5 column (J&W Scientific, Folsom, CA); He carrier gas, 5 mL/min; detector base at 260 °C; ELCD reactor at 900 °C; H_2 reaction gas with nickel tube; solvent, *n*-propanol (25 μL/min); detector in halogen mode, high sensitivity. The GC was programmed from 130 to 225 °C at 5 °C/min and then to 250 °C at 25 °C/min (hold for 15 min). Polar column conditions were as follows: 30 m × 0.53 mm i.d., 1 μm DB-Wax column (J&W Scientific); GC programmed from 150 to 230 °C at 5 °C/min (hold for 15 min); He carrier gas, 6 mL/min; detector base at 240 °C. Other conditions remained the same. All injections were manual, on-column at 0.1 μL of the neat sample. Three peaks in higher concentration (chlorohydrin peaks 1−3 on DB-Wax) were chosen to study (**Figures 1** and **2**).

Quantification Utilizing GC-ELCD. 2,3-Dichloroaniline (Acros Organics, Pittsburgh, PA) was used as an internal standard in quantitative studies of peak 1 on the DB-Wax column. A linear response was observed from 0.7 to 35 ppm for the purified chlorohydrin peak 1 and from 0.4 to 30 ppm for the internal standard. *d*-Limonene (99.5% pure from a local flavor company) was used as the solvent for peak 1. Isopropyl alcohol (Acros) was used as the solvent for the internal standard. The internal standard was spiked at similar concentrations as the analyte. Oils were diluted with isopropyl alcohol when necessary to remain within the range of linear response. A relative response factor was calculated to be 0.59.

GC-FID Analysis. The purity of chlorohydrins during recovery and isolation procedures was monitored by GC-FID as follows: Hewlett-Packard 5890; 30 m × 0.53 mm i.d., 1 μm DB-Wax column, splitless

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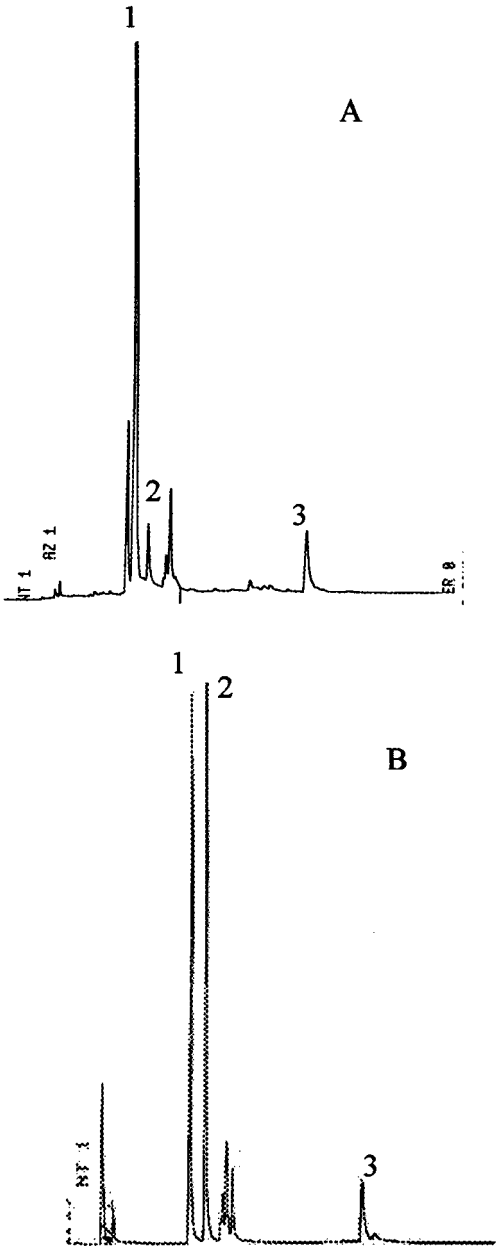

Figure 1. GC-ELCD chromatogram using a DB-Wax column of (A) a cold-pressed oil containing chlorohydrins **1**–**3** and (B) a limonene–bleach reaction mixture containing chlorohydrins **1**–**3**.

insert; programmed from 100 to 230 C at 5 °C/min, hold for 10 min; injector at 230 °C; detector at 240 °C; H_2 carrier gas, 5.25 mL/min. Retention indices (RI) for the three chlorohydrins were determined using C-12 through C-26 hydrocarbons on a DB-Wax column (RI peak 1 = 1994; RI peak 2 = 2049; RI peak 3 = 2530) and C-10 through C-22 on a DB-5 column (RI peak 1 = 1342; RI peak 2 = 1340; RI peak 3 = 1609). Calibration curves were established by fitting to a polynomial regression equation ($R^2 = 0.9999$).

Reaction Products of Bleach and Chloramine-T. Chlorohydrin synthesis to prepare standards was performed by two different methods, one using household bleach (5.25% sodium hypochlorite) and the other, chloramine-T (Fisher Scientific). High-purity *d*-limonene was further purified by silica gel adsorption chromatography to >99.5% purity (GC-FID peak area). HOCl (200 ppm) was prepared from common household bleach using pH 6.2 phosphate buffer. Purified *d*-limonene (200 mL) and the HOCl buffered solution (200 mL) were placed in a beaker with a stir bar and mixed for 0.5 h, and the bottom aqueous layer was removed using a separatory funnel. The reacted limonene layer was rinsed three times with 200 mL of purified water to remove

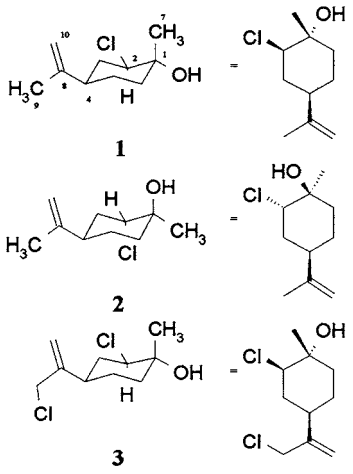

Figure 2. Structures of chlorohydrins (1*R*,2*R*,4*R*)-2-chloro-8-*p*-menthen-1-ol (**1**), (1*S*,2*S*,4*R*)-2-chloro-8-*p*-menthen-1-ol (**2**), and (1*R*,2*R*,4*R*)-2,9-dichloro-8-*p*-menthen-1-ol (**3**).

residual chlorine and dried with anhydrous sodium sulfate. In other experiments, similar reactions were conducted with 2000 ppm solutions of HOCl.

Total residual chlorine in bleach solutions was quantified by the diethyl-*p*-phenylenediamine (DPD) colorimetric EPA Method 330.5, using potassium permanganate standards. Modifications to the method were the following: DPD tablets no. 4 (Orbeco, Farmingdale, NY), which contained all of the necessary reagents (DPD, potassium iodide), were used for total residual chlorine analysis; 10 mL samples were placed in 15 mL test tubes, rotated on a chemistry mixer (Fisher Scientific) for 10 min. Absorbance was measured at 515 nm.

To increase yields of limonene chlorohydrins, synthesis was also undertaken by a reaction method using chloramine-T (*10*). *d*-Limonene (1 mol) was reacted with chloramine-T (1 mol) suspended in 1:1 acetone/H_2O (800 mL). After reaction, the organic phase was separated; the aqueous phase was extracted with pentane (3 × 30 mL), and the pentane extracts were added to the organic phase, dried with anhydrous sodium sulfate, and solvent evaporated in vacuo. Reaction products were concentrated and purified by silica gel column chromatography and preparative HPLC.

Concentration of Chlorohydrins in Cold-Pressed Orange Oil. Concentration of chlorohydrin compounds was accomplished using adsorption chromatography (*11*, *12*). Silica gel (22 g), 200−425 mesh, Davisil grade 633 type 60A, was applied as a slurry in hexane to a 2 cm diameter column, column height = 18 cm, volume = 56 mL. A commercial cold-pressed orange oil confirmed to contain chlorinated compounds was applied to the column until breakthrough of chlorinated compounds (by GC-ELCD) occurred (300 mL of oil). The column was then rinsed with hexane (1 L) to remove the remaining limonene and oil. Chlorohydrin compounds were eluted with 10% ethyl acetate in hexane until none were detected in the fractions collected (8 × 50 mL fractions). Fractions containing similar peaks were combined, and the solvent was removed by rotary evaporator.

Concentration of Synthesized Chlorohydrins. *Limonene−Bleach.* Silica gel (20 g), 200−425 mesh, Davisil grade 633 type 60A was applied to a 2 cm column as a slurry in 99.5% pure *d*-limonene. Column height was 16 cm, volume = 50 mL. The reaction products (180 mL) from the limonene−bleach reaction were applied to the column. To conserve resources, the limonene that was eluted from the column was reused for additional reactions with HOCl and continually reapplied to the column until breakthrough of chlorinated compounds of interest occurred. In total, 730 mL of reaction product was applied to the column. The column was rinsed with hexane (8 column volumes) to remove the remaining limonene. Chlorohydrins were eluted with 10% ethyl acetate/hexane (400 mL) followed by 15% ethyl acetate/hexane (200 mL). Fractions containing the same GC-ELCD peaks were combined, and solvent was removed using a rotary evaporator.

In another experiment in which a 2000 ppm HOCl solution was reacted with limonene, a total of 963 mL of reaction product was applied

Table 1. ^{13}C and ^{1}H NMR Data for (1*R*,2*R*,4*R*)-2-Chloro-8-*p*-menthen-1-ol (**1**) (Recorded in C_6D_6)

C	δ_C (mult)	δ_H (mult)	COSY
1	73.0 (s)		
2	71.5 (d)	3.8 (dd, J = 4, 12.5)	H-3α, H-3β
3	39.7 (t)[a]	1.50 (m)	H-2, H-3α, H-4
		1.96 (m)	H-2, H-3β, H-4
4	45.6 (d)	1.62 (m)	H-3α, H-3β, H-5α, H-5β
5	28.6 (t)	1.00 (m)	H-4, H-5α, H-6α, H-6β
		1.35 (m)	H-4, H-5β, H-6α, H-6β
6	39.3 (t)[a]	1.38 (m)	H-6β, H-5α, H-5β
		1.74 (m)	H-6α, H-5α, H-5β
7	21.0 (q)[b]	1.10 (s, 3H)	
8	148.1 (s)		
9	21.1 (q)[b]	1.45 (br s, 3H)	H-10, H-10′
10	110.0 (t)	4.59 (br s)	Me-9
		4.67 (br s)	Me-9

[a] Assignments may be reversed. [b] Assignments may be reversed.

to a 25 g silica gel column and a similar procedure for elution of chlorinated compounds was followed.

Limonene−Chloramine-T. Silica gel (10 g), 200−425 mesh, was applied to a 2 cm column as a slurry in hexane (volume = 25 mL). Chloramine-T reaction products (2 mL) were applied, and the column was rinsed with hexane (400 mL) to remove the remaining limonene and other less polar compounds. Chlorohydrins were eluted sequentially with 5% (300 mL), 10% (100 mL), and then 20% (50 mL) ethyl acetate/hexane. Fractions containing the same GC-ELCD peaks were combined, and solvent was removed using a rotary evaporator.

HPLC Purification of Chlorohydrins. *Cold-Pressed Oil.* Crude concentrated oil fractions were purified using an HPLC system consisting of an LDC Analytical (Riviera Beach, FL) programmable isocratic pump, a fluid metering pump, and a refractive index detector (LDC Analytical). A 25 cm × 1 cm i.d., particle size = 8 μm, Dynamax 60 Å silica preparative column (Rainin Instrument Co., Woburn, MA) was installed. The solvent system consisted of 9% ethyl acetate/91% hexane at 4 mL/min.

Reaction Products from Bleach and Chloramine-T. Utilizing the HPLC system described previously, chlorohydrin peak 1 was purified using 1% isopropyl alcohol (IPA)/hexane at 4 mL/min, peak 2 was purified with 0.9% IPA/hexane at 3.5 mL/min, and peak 3 purified with 3% IPA/hexane at 4 mL/min.

Mass Spectrometric Analysis. A Hewlett-Packard 5971 MSD (70 eV) connected to a Hewlett-Packard 5890 gas chromatograph was used for low-resolution EI mass spectral analysis. Polar column conditions were as follows: 60 m × 0.25 mm i.d., 0.25 μm DB-Wax column (J&W Scientific), splitless insert. The GC was programmed from 100 to 230 °C at 5 °C/min (hold for 5 min); H_2 carrier gas at 1.17 mL/min; injector at 230 °C, detector at 250 °C. Nonpolar conditions: 60 m × 0.25 mm i.d., 0.25 μm DB-35 (J&W Scientific); H_2 carrier gas at 1 mL/min; injector at 250 °C, detector at 280 °C; GC programmed at 60 °C (hold for 1 min) to 250 °C at 5 °C. min (hold for 5 min).

High-resolution electron impact mass spectra were acquired using a Kratos MS 50 RFA spectrometer.

NMR Analysis. A Bruker 300 MHz NMR spectrometer with an ARX console and an Avance magnet was used for NMR analyses.

(1*R*,2*R*,4*R*)-2-Chloro-8-*p*-menthen-1-ol (**1**): white crystalline powder; mp 63−64 °C; IR (film) 3330, 2950 cm^{-1}; see **Table 1** for NMR spectroscopic shifts; EI-HRMS (M^+ calculated for $C_{10}H_{17}O^{35}Cl$ 188.09679; found, 188.09705).

(1*S*,2*S*,4*R*)-2-Chloro-8-*p*-menthen-1-ol (**2**): clear viscous liquid; IR (film) 3440, 2940 cm^{-1}; see **Table 2** for NMR spectroscopic shifts; EI-HRMS M^+ calculated for $C_{10}H_{17}O^{35}Cl$ 188.09679; found 188.09743).

(1*R*,2*R*,4*R*)-2,9-Dichloro-8-*p*-menthen-1-ol (**3**): white crystalline powder, mp not determined; IR (film) 3430, 2940, 2360; see **Table 3** for NMR spectroscopic shifts; CIMS M^+ 240, 242, 244 $[M + NH_4]^+$; EI-HRMS M^+ calculated for $C_9H_{13}O^{35}Cl_2$ $[M - CH_3]^+$ 207.03435; found 207.03467.

Table 2. ^{13}C and ^{1}H NMR Data for (1*S*,2*S*,4*R*)-2-Chloro-8-*p*-menthen-1-ol (**2**) (Recorded in C_6D_6)

C	δ_C (mult)	δ_H (mult)	COSY
1	71.6 (s)		
2	66.1 (d)	3.73 (dd, J = 4, 4 Hz)	H-3α, H-3β
3	35.5 (t)	1.84 (m)	H-2, H-3α, H-4
		2.15 (m)	H-2, H-3β, H-4
4	38.1 (d)	2.48 (m)	H-3α, H-3β, H-5α, H-5β
5	26.7 (t)	1.48 (m)	H-4, H-5α, H-6α, H-6β
		1.60 (m)	H-4, H-5β, H-6α, H-6β
6	33.4 (t)	1.30 (m)	H-6β, H-5α, H-5β
		1.78 (m)	H-6α, H-5α, H-5β
7	29.1 (q)	1.15 (s, 3H)	
8	149.3 (s)		
9	21.5 (q)	1.62 (br s, 3H)	H-10, H10′
10	109.9 (t)	4.78 (br s, 2H)	Me-9

Table 3. ^{13}C and ^{1}H NMR Data for (1*R*,2*R*,4*R*)-2,9-Dichloro-8-*p*-menthen-1-ol (**3**) (Recorded in C_6D_6)

C	δ_C (mult)	δ_H (mult)	COSY
1	72.7 (s)		
2	71.0 (d)	3.67 (dd, J = 4, 12.5 Hz)	H-3α, H-3β
3	39.8 (t)[a]	1.38 (m)	H-2, H-3α, H-4
		1.90 (m)	H-2, H-3β, H-4
4	41.1 (d)	1.85 (m)	H-3α, H-3β, H-5α, H-5β
5	28.9 (t)	0.90 (m)	H-4, H-5β, H-6α, H-6β
		1.36 (m)	H-4, H-5α, H-6α, H-6β
6	39.1 (t)[a]	1.44 (m)	H-6β, H-5α, H-5β
		1.64 (m)	H-6α, H-5α, H-5β
7	20.9 (q)	1.05 (s, 3H)	
8	148.1 (s)		
9	47.5 (t)	3.52 (s, 2H)	H-10, H-10′
10	114.1 (t)	4.57 (br s)	H-9
		4.80 (br s)	

[a] Assignments may be reversed.

RESULTS AND DISCUSSION

Detection of Chlorohydrins. Polar column GC conditions produced the best separation of the three chlorohydrins produced by the reaction of limonene with HOCl (**Figure 1**), whereas chlorohydrin peaks 1 and 2 appeared as a poorly resolved doublet using a nonpolar column (not shown). After HPLC purification of the individual peaks, it was determined that the elution order of the polar GC column peaks 1 and 2 was reversed on the nonpolar GC column.

Concentration and Purification of Cold-Pressed Orange Oil Chlorohydrins. *Concentration.* During this study, 52 commercial citrus oils were quantitatively analyzed, using 2,3-dichloroaniline as the internal standard, for the presence of chlorohydrin peak 1. Of the oils tested, 35 were found to contain <2 ppm, 15 oils contained between 3 and 25 ppm, one commercial folded orange oil contained 160 ppm, and an orange carbonyl fraction contained 850 ppm of peak 1. We concentrated the chlorohydrins from one of the more contaminated samples of commercial cold-pressed orange oil estimated to contain 25 ppm of chlorohydrin peak 1. Chlorohydrin-containing fractions from ~1200 mL of this cold-pressed oil were concentrated and purified ~1200-fold.

It was difficult to separate chlorohydrins in cold-pressed oils from nonvolatile pigments and oxygenated polar components retained on the silica column. However, column elution with 10% ethyl acetate/hexane resulted in the best separation of the three chlorohydrins. Peaks 1 and 2 eluted together in the first three fractions. Peak 3 eluted in fractions 3−8.

Purification. Concentrated fractions produced by silica column adsorption were further purified by HPLC. Separation of

the three limonene chlorohydrins was accomplished with recoveries sufficient for GC-FID and mass spectral analyses.

The three chlorohydrins found in cold-pressed oil were compared with the three *d*-limonene reaction products. Similar mass spectra and similar retention times on both nonpolar and polar GC columns were presumptive evidence that they are identical compounds. Thus, no further purification steps were conducted on the cold-pressed oil fractions. Instead, we focused on purification and identification of the synthetic limonene chlorohydrins.

Syntheses of Terpene Chlorohydrins. Reactions of *d*-limonene with unbuffered (pH 9.6) and buffered (pH 6.2) HOCl were compared. Formation of **3** was observed only in the buffered reaction. A weak acid, HOCl dissociated readily at higher pH, whereas at pH 6.2, ~5% of the HOCl was dissociated (*13*). Thus, we performed all reactions in pH 6.2 buffer. Reactions conducted with a 2000 ppm HOCl solution as the reactant formed higher concentrations of chlorohydrins. Formation of peak 3 was especially dependent on higher concentrations of HOCl. Accordingly, peak 3 was detected only in highly contaminated cold-pressed oils.

Data supported the pH effects as follows: GC-ELCD peak areas at pH 9.6 (unbuffered) were peak 1 = 100,000, peak 2 = 40,000, and no peak 3, whereas at pH 6.2 (buffered), peak 1 = 130,000, peak 2 = 120,000, and peak 3 = 40,000. Commercial oils with large peak 2 concentrations could be explained, because careful water chlorination involves proper pH water treatment. Some processors probably monitor and adjust water pH more carefully for more effective chlorination.

Polar column GC-ELCD chromatograms of a highly contaminated, unconcentrated cold-pressed orange oil (**Figure 1A**) and limonene−HOCl reaction product (**Figure 1B**) indicated that more of peak 2 was present in the limonene reaction mixture compared to the cold-pressed oil shown in **Figure 1A**. Our analyses indicated that some cold-pressed oils contained higher amounts of peak 2 than of peak 1.

Purification of Synthesized Chlorohydrins. Batches of several reaction mixtures were combined to obtain sufficient amounts of chlorohydrins for purification and structure elucidation. HPLC conditions were modified to increase the resolution and separation of the compounds of interest. After initial attempts at HPLC purification using ethyl acetate/hexane (9:91) eluent, it was found that no further purification could be achieved after concentration and re-injection. Therefore, the solvent system was changed to 2-propanol/hexane. Flow rate and percent 2-propanol were optimized to achieve the best separation for each peak, with a result of ⩾94% purity for all three chlorohydrins. Fractions from different experiments were combined to accumulate the required amounts for NMR analyses.

NMR Analyses and Structure Elucidation. *Chlorohydrin 1*. (1*R*,2*R*,4*R*)-2-Chloro-8-*p*-menthen-1-ol (**1**, **Figure 2**) was isolated as a white crystalline powder (47 mg; purity = 95% by GC-FID), from reaction mixtures of *d*-limonene with HOCl or chloramine-T reagent. The small crystals observed on the sides of the glass vial in which **1** was stored were not suitable for X-ray crystallographic analysis. Retention indices for **1** were determined on nonpolar and polar GC columns. Chlorohydrin **1** gave a parent ion in the EI-HRMS at *m*/*z* 188.09705 Da, consistent with a molecular formula of $C_{10}H_{17}O^{35}Cl$ (ΔM + 0.26). Additionally, the low-resolution electron impact mass spectrum showed molecular ions at *m*/*z* 188 and 190 with relative abundances of approximately 3:1, respectively, characteristic of ions containing chlorine isotopes. Two degrees of unsaturation indicated **1** was produced from *d*-limonene by the loss of either the ring or a double bond.

The ^{1}H NMR spectrum of **1** contained singlets that could be assigned to the two tertiary methyl groups found in the menthene carbon skeleton. A broad singlet at δ 1.45 (3H) was assigned to Me-9, whereas a singlet at δ 1.10 was shielded relative to the corresponding methyl resonance in limonene, indicating that in **1** it was situated on a saturated, fully substituted carbon. A pair of broad singlets at δ 4.67 and 4.59 were assigned to H10 and H10′.

The ^{13}C NMR spectrum (**Table 1**) contained 10 carbon signals, consistent with the molecular formula. A single pair of olefinic carbon resonances at δ 148 (s) and 110 (t) indicated that **1** contained only one double bond. An APT NMR experiment provided information about the multiplicities of the carbons present in **1**. The chemical shifts of the olefinic carbon resonances as well as the triplet multiplicity of the resonance at δ 110, determined from the APT spectrum, indicated that **1** contained an exocyclic double bond.

Carbon NMR signals at δ 71 (s) and 73 (d) were consistent with the reaction of hypohalous acid with the internal double bond of limonene to form a halohydrin. An HMQC experiment was acquired to identify all protons and ^{13}C nuclei that are connected by a single bond. From the results of APT and HMQC experiments, the resonance at δ 73 was assigned to C-1, bound to a hydroxyl group, whereas the signal at 71 ppm was assigned to C-2, which bore the chlorine substituent. This assignment was consistent with Markovnikov's rule and with literature reports of chlorohydrins formed from reactions of HOCl with alkenes (*4*, *5*). The HMQC spectrum of **1** contained a correlation from the ^{13}C resonance at δ 71 to the ^{1}H signal at 3.8 ppm (dd, 1H, J = 4, 12.5 Hz), and accordingly the ^{1}H resonance was assigned to H-2. The isopropylene substituent at C-4 was tentatively assigned the β configuration. From the observation of a diaxial trans coupling constant between H-2 (δ 3.8, dd, J = 4, 12.5 Hz) and H-3$_{ax}$, H-2 was assigned the α and the chlorine substituent the β configuration. COSY and HMQC correlations allowed us to elaborate an isolated proton spin system consisting of −CHCl−CH$_2$−CH−CH$_2$−CH$_2$ (**Table 1**). To observe two- and three-bond coupling between the protons and ^{13}C nuclei present in **1**, a COLOC spectrum was acquired. The COLOC contained correlations from the H10 and H10′ resonances at δ 4.67 and 4.59 to C-4 (45.2 ppm) and C-9 (20.7 ppm).

The ^{1}H NMR spectrum of **1** in pyridine-d_5 was compared to the spectrum in $CDCl_3$ to determine the configuration of the hydroxyl group (*14*). In this experiment, the pyridine nitrogen exhibited selective hydrogen bonding to the hydroxyl group of **1**, resulting in a selective anisotropic effect of the pyridine upon the protons neighboring the hydroxyl group, and consequently a significant downfield shift (≥0.3 ppm) in the resonances of these protons was observed. A large $\Delta\delta$ for the H-2 resonance, from 4.0 to 4.35 ppm (+0.35 ppm), was observed, allowing assignment of the equatorial α configuration to the hydroxyl substituent (*14*). The results were consistent with a diequatorial trans configuration for the halohydrin substitution of **1**.

NOE experiments provided information concerning the spatial proximity of protons within chlorohydrin **1**. Upon irradiation of the H-2 resonance (δ_H 3.8), nuclear Overhauser enhancements (NOE) were observed at δ 1.96 (H-3$_\alpha$), δ 1.62 (H-4), and δ 1.38 (H-6$_\alpha$). Upon irradiation of the ^{1}H NMR signal at δ 1.62 (H-4), a reverse NOE was observed at 3.8 ppm (H-2). These results indicated that **1** was in a chair conformation and the isopropenyl substituent was in the equatorial configuration. The

results of the NOE experiments in conjunction with the ^{1}H NMR shifts observed in pyridine-d_5 and based on the known stereochemistry of *d*-limonene [(4*R*)-1,8-*p*-menthadiene] permitted assignment of the stereochemistry of **1** as (1*R*,2*R*,4*R*)-2-chloro-8-*p*-menthen-1-ol.

Chlorohydrin 2. Fractions from reactions of *d*-limonene with chloramine-T and bleach were pooled to yield (1*S*,2*S*,4*R*)-2-chloro-8-*p*-menthen-1-ol (**2**, **Figure 2**) as a clear viscous liquid (36 mg, purity = 95% by GC-FID). Retention indices were determined on nonpolar and polar GC columns. Chlorohydrin **2** gave a parent ion in the EI-HRMS at *m*/*z* 188.09734 Da, consistent with a molecular formula of $C_{10}H_{17}O^{35}Cl$ (ΔM + 0.54).

The ^{1}H and ^{13}C NMR spectra of **2** were very similar to the spectra of **1** (**Table 2**). The NMR spectra of **2** indicated that it, too, was a cyclic, monoterpene halohydrin with a single exocyclic double bond. A significant difference was observed in the ^{1}H NMR spectrum of **2** compared with **1**, in that the H-2 resonance of **2** at δ 3.75 was a doublet of doublets with coupling constants of 4 Hz, indicating that the H-2 proton was equatorial, whereas the chlorine attached at C-2 was in the axial position. The COSY spectrum of **2** contained correlations from the resonance at δ 4.01 (in $CDCl_3$, H-2) to resonances at 2.19 and 1.89 ppm, which were attributed to H-3α and H-3β, respectively. Analysis of the COSY and HMQC spectra allowed assignment of all of the carbon and proton resonances in $CDCl_3$ and pyridine-d_5. The resonances at δ 1.89 and 1.56, which were assigned to H-3β and H-5β, respectively, shifted downfield to 2.65 and 2.00 ppm in pyridine-d_5. These results were consistent with assignment of β configuration to the hydroxyl substituent and, consequently, with diaxial trans configuration for the halohydrin substitution of **2**. This assignment was supported by our observation that lower concentrations of **2** were found in oils produced using unbuffered HOCl. As reported by Kopperman and co-workers (*4*), at higher pH, diaxially substituted chlorohydrins undergo facile elimination of chloride to produce the corresponding epoxide. The isopropenyl substituent was determined to be in the equatorial configuration because the coupling of H-4 was identical to that observed in **1**. Furthermore, the ^{1}H NMR signal for H-4 of **2**, at δ 2.48, was shifted downfield relative to the resonance for H-4 of **1** (1.62 ppm). This was attributed to the γ effect of the axial chloride substituent of **2**. On the basis of the known stereochemistry of *d*-limonene and by comparing the NMR data to literature values, **2** was identified as (1*S*,2*S*,4*R*)-2-chloro-8-*p*-menthen-1-ol (*7*, *9*).

Chlorohydrin 3. A hypochlorous acid (2000 ppm) reaction with *d*-limonene yielded (1*R*,2*R*,4*R*)-2,9-dichloro-8-*p*-menthen-1-ol (**3**, **Figure 2**), a white crystalline powder (11 mg, purity = 94% by GC-FID). Retention indices for **3** were determined on nonpolar and polar GC columns. Chlorohydrin **3** gave an ion fragment in the EI-HRMS at *m*/*z* 207.03467 ($C_9H_{13}O^{35}Cl_2$, $[M - CH_3]^+$, ΔM + 0.32 mmu). No molecular ion was observed in the EI-HRMS. However, a chemical ionization mass spectrum in the positive mode using ammonia gas contained molecular ions at *m*/*z* 240, 242, and 244 ($M^+ + NH_4$), appropriate for a molecular formula of $C_{10}H_{16}OCl_2$, with a relative isotope abundance of approximately 9:6:1, typically seen with molecules containing two chlorine atoms.

The ^{1}H and ^{13}C NMR spectra were very similar to the spectra of **1** (**Table 3**). The most obvious differences in the proton NMR spectrum were the absence of the methyl resonance at δ 1.10 and the presence of a broad singlet at 3.52 ppm, which integrated for two protons. The HMQC spectrum contained a correlation from the proton resonance at δ 3.52 to a carbon signal at 47.5 ppm. In addition, the broad singlet at δ 3.52 showed a COSY correlation to the olefinic protons at 4.80 and 4.57 ppm (**Table 3**). This was evidence that the C-9 methyl had been replaced by a chloromethylene. It is hypothesized that both double bonds of limonene reacted with hypochlorous acid to form the dihalohydrin with subsequent elimination of the hydroxyl group at C-10 to form **3**.

Upon comparison of ^{1}H NMR spectra run in $CDCl_3$ and pyridine-d_5, H-2 showed a significant downfield shift from 4.05 to 4.37 ppm consistent with the diequatorial trans configuration for the halohydrin substituents, identical to **1**. These results permitted assignment of the stereochemistry of **3** as (1*R*,2*R*,4*R*)-2,9-dichloro-8-*p*-menthen-1-ol.

In this investigation, three terpene chlorohydrins found in cold-pressed orange oil were isolated and identified using GC-MS and NMR analyses. GC-ELCD analyses of these compounds in cold-pressed oils were simple, requiring only direct injection of a 0.1 μL neat sample. Formation of the monoterpene chlorohydrins was determined to be the result of a reaction of *d*-limonene, the major component of cold-pressed orange oil with HOCl, found in chlorinated treatment water used in the oil recovery process. Pilot and processing plant studies are underway to study the variables affecting the formation of these compounds during the manufacture of citrus essential oils.

ABBREVIATIONS USED

APT, attached proton test; COLOC, correlation via long-range couplings; COSY, correlation spectroscopy; GC-ELCD, gas chromatography−electrolytic conductivity detector; GC-FID, gas chromatography−flame ionization detector; HMQC, heteronuclear multiple quantum correlation; HOCl, hypochlorous acid; HPLC, high-performance liquid chromatography; MS, mass spectrometry; NOE, nuclear Overhauser enhancement; 2D NMR, two-dimensional nuclear magnetic resonance.

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LITERATURE CITED

(1) Orme, J.; Mullin, C. S.; Ohanian, E. V. Health effects of disinfectants and disinfection by-products: a regulatory perspective. In *Water Chlorination: Chemistry, Environmental Impact, and Health Effects*; Jolley, R. L., Codie, L. W., Johnson, J. D., Katz, S., Minear, R. A., Mattice, J. S., Jacobs, V. A., Eds.; Lewis Publishers: Chelsea, MI, 1990; Vol. 6. pp 75−86.

(2) Hegde, G. S.; Vogel, M. K.; Saddler, J.; Hrinyo, T.; Rockwell, N.; Haynes, R.; Oliver, M.; Wolinsky, J. The reaction of hypochlorous acid with olefins. A convenient synthesis of allylic chlorides. *Tetrahedron Lett*. **1980**, *21*, 441−444.

(3) Wolinsky, J.; Vogel, M. K. A reinvestigation of the reaction of a-pinene with hypochlorous acid. *J. Org. Chem*. **1977**, *42*, 249−253.

(4) Kopperman, H. L.; Hallcher, R. C., Sr.; Riehl, A.; Carlson, R. M.; Caple, R. Aqueous chlorination of α-terpineol. *Tetrahedron* **1976**, *32*, 1621−1626.

(5) Stuthridge, T. R.; Wilkins, A. L.; Langdon, A. G. Identification of novel chlorinated monoterpenes formed during kraft pulp bleaching of *Pinus radiata*. *Environ. Sci. Technol.* **1990**, *24*, 903−908.
(6) Wylde, R.; Teulon, J. Les époxydes du limonène: synthèse et réactivité. *Bull. Soc. Chim. Fr.* **1970**, *2*, 758−764.
(7) Bovicelli, P.; Mincione, E.; Ortaggi, G. Chemo and regioselective opening of ethereal carbon−oxygen bonds by monochloroborane-dimethylsulfide. *Tetrahedron Lett.* **1991**, *32*, 3719−3722.
(8) Mukhamedova, L. A.; Nasybullina, F. G.; Kudryavtseva, M. I. Reaction of *p*-menthadiene dioxide with hydrogen chloride. *Arbuzov Inst. Org. Phys. Chem., Acad. Sci. USSR* **1979**, *28*, 791−795.
(9) Arbuzov, B. A.; Isaeva, Z. G.; Bikbulatova, G. Sh.; Semakhina, N. I. The reaction of α-3,4-epoxycarene with hydrogen chloride. *Arbuzov Inst. Org. Phys. Chem., Acad. Sci. USSR* **1972**, *207*, 909−912.
(10) Damin, B.; Garapon, J.; Sillion, B. A convenient synthesis of chlorohydrins using chloramine T. *Synthesis* **1981**, 362−363.
(11) Kirchner, J. G.; Miller, J. M. Preparation of terpeneless essential oils. *Ind. Eng. Chem.* **1952**, *44*, 318−325.
(12) Braverman, J. B. S.; Solomiansky, L. Separation of terpeneless essential oils by the chromatographic method. *Perfum. Essent. Oil Record* **1957**, 284−287.
(13) White, G. C. Chemistry of chlorination. In *Handbook of Chlorination*; Van Nostrand Reinhold: New York, 1972; pp 182−228.
(14) Demarco, P. V.; Farkas, E.; Doddrell, D.; Mylari, B. L.; Wenkert, E. Pyridine-induced solvent shifts in the nuclear magnetic resonance spectra of hydroxylic compounds. *J. Am. Chem. Soc.* **1968**, *90*, 5480−5486.

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