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NEUTRAL COMPOUNDS FROM MALE CASTOREUM OF NORTH AMERICAN BEAVER, *Castor canadensis*

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Abstract—North American beavers (*Castor canadensis*) mark their territories with castoreum, the strong-smelling paste in their castor sacs. In their own territories, beavers respond with scent marking to experimental scent marks that consist of strange castoreum (or selected components). In part, the unique odor of castoreum is due to large amounts of phenolic compounds and neutral compounds. Purified neutral compounds were analyzed by GC, GC-MS, and NMR; identities of the neutral compounds were confirmed by comparing the properties of authentic compounds with those of the isolated compounds. We identified 13 neutral compounds that had not been reported before for castoreum. Most of these are oxygen-containing monoterpenes. Of the nine neutral compounds reported by Lederer (1949), only three are confirmed in our analysis; the other six neutral compounds are either absent or are not volatile enough to be detected by our methods. Eight compounds—6-methyl-1-heptanol, 4,6-dimethyl-1-heptanol, isopinocamphone, pinocamphone, two linalool oxides, and their acetates—were synthesized for structure identification and bioassays.

Key Words—beaver, *Castor canadensis*, castoreum, neutral compounds, monoterpenes, co-injection, fractionation, identification, synthesis, territory marking.

INTRODUCTION

Castoreum is a strong-smelling brown paste contained in the paired castor sacs of both the Old and New World beaver (*Castor fiber* and *Castor canadensis*,

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0098-0331/95/1100-1745$07.50/0

respectively). Distinguished from the castor sacs are a pair of anal glands, termed "oil sacs" by trappers. Castoreum consists of phenolic, neutral, basic, and acidic components.

As a result of several field studies in recent years, the biological role of castoreum in the social life of beaver is now better understood (Hodgdon, 1978; Bollinger, 1980; Müller-Schwarze and Heckman, 1980; Svendsen, 1980; Houlihan, 1989; Schulte et al., 1994). Beaver deposit castoreum and anal gland secretion on mud piles that they build at the banks of their ponds. The scent marks applied to these "scent mounds" are territorial signals (Houlihan, 1989). Furthermore, unoccupied beaver habitat is less likely to be colonized if it is scented with artificial scent mounds (Welsh and Müller-Schwarze, 1989).

Castoreum has been bioassayed in behavioral experiments several times. Resident beaver respond to experimental scent mounds by approaching, going on land, sniffing the mound, pawing it, and often marking the sample (Hodgdon, 1978; Bollinger, 1980; Müller-Schwarze and Heckman, 1980; Svendsen, 1980). Castoreum fractions also elicit these behavioral activities (Müller-Schwarze et al., 1986; Svendsen and Huntsman, 1988), but less so with progressive fractionation. The activity disappears completely well before the level of single compounds is reached (Müller-Schwarze et al., 1986).

Because bioassays of fractions led to a level of fractionation with no demonstrable biological activity, single compounds known to be constituents of castoreum and their mixtures were tested in subsequent experiments (Houlihan, 1989; Müller-Schwarze and Houlihan, 1990; Schulte et al., 1994). Several compounds were active, albeit at a very low level. The activity of mixtures was intermediate between castoreum and single compounds (Müller-Schwarze and Houlihan, 1990; Müller-Schwarz, 1992, Schulte et al., 1994).

This chemical study was prompted by these and other ongoing behavior investigations. It is imperative to know the composition of castoreum from the specific study populations and to bioassay any compounds hitherto unknown for castoreum.

The composition of castoreum has been investigated several times. Walbaum and Rosenthal (1927) first identified some phenolic and neutral compounds from castoreum; the neutral compounds were acetophenone, borneol, and benzyl alcohol. Later, Stevens (1943) separated some neutral red oil from dried beaver castors called American musk, but he did not report any further isolation work. Lederer (1946, 1949) identified nine neutral compounds from castoreum: benzyl alcohol, cholesterol, β-cholestanol, mannitol, *cis*-5-hydroxytetrahydroionol, borneol, acetophenone, 4-methoxyacetophenone, and cholesteryl oleate. Valenta et al. (1960) isolated *cis*-cyclohexane-1,2-diol from beaver castoreum. Maurer and Ohloff (1976) isolated nitrogen containing compounds from castoreum. Newly found phenolic components were reported in Tang et al. (1993).

The anal gland contains chemically very different compounds. Many C_{15}–C_{22} carboxylic acids and their waxy esters from the anal gland secretion were characterized by Grønneberg (1978) and Grønneberg and Lie (1984) using thin layer chromatography (TLC), gas chromatography (GC), and mass spectroscopy (MS). No further work concerning neutral compounds has been reported since.

We have reinvestigated the neutral compounds extracted from male castoreum. The compounds were isolated by packed column GC fractionation and analyzed by capillary GC, gas chromatography–mass spectrometry (GC-MS), capillary GC coinjection, nuclear magnetic resonance (NMR) spectrometry and by comparing the spectra of the unknown components with those of synthesized compounds. We identified 16 neutral compounds, 13 of which had not been known from castoreum.

METHODS AND MATERIALS

Chemicals. The solvents used for extraction and for flash column chromatography were reagent grade. Solvents used for capillary GC were HPLC grade. Anhydrous tetrahydrofuran (THF) was dried over 4 Å molecular sieves. Ethylene oxide was purchased from Eastman company, dried over sodium, and distilled before use. Linalool was purchased from Kodak. All other reagents were purchased from Aldrich Chemical Company.

Instrumentation. Analytical capillary GC was performed on a Varian 3700 gas chromatograph equipped with a splitless injector, fused silica column, and a flame ionization detector (FID). Standard capillary GC conditions for all identification work used the following temperature program: 40°C (3 min), 3°C/min, 210°C (30 min). Carrier and makeup gas for capillary GC work was nitrogen. Capillary GC analyses were carried out on one of two columns: (A) DB-1, 30 m × 0.25 mm; (b), FFAP column, 50 m × 0.25 mm.

Capillary GC coinjection was conducted by taking 0.5 ml of a fraction (5–10 ng/μl) and adding 1 μg of a standard to it; 2 μl of this mixture were injected onto the capillary GC. The unknown compounds were coinjected with the same standards on one or two capillary GC columns (one polar and one nonpolar), except for nojigiko alcohol and junenol because they are not commercially available and their syntheses are beyond the scope of this investigation.

Semipreparative-scale packed-column GC fractionations were performed on a Varian 2700 aerograph instrument. All GC fractions were collected in glass capillary tubes (3 mm × 30 cm) using a Brownlee-Silverstein thermal gradient collector (Brownlee and Silverstein, 1968). The purity of the isolated fractions was checked by capillary GC on column A. The pure fractions were then char-

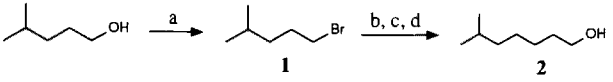
acterized by GC-MS and NMR. The following packed columns were used to purify the crude extract: column C, OV-101 (5%) on Chromasorb W, 3 m × 10 mm OD; column D, Carbowax 20 M (4%) on Chromasorb G, 6 m × 6 mm, temperature program: 100°C (0 min), 4°C/min, 230°C (5 min).

Mass spectra were obtained using a Finnigan 4500 automated gas chromatograph/EI-CI mass spectrometer system (GC-MS). All spectra were obtained at 70 eV. Proton NMR (^{1}H NMR) spectra were obtained using a Bruker AMX-300 (300 MHz). All spectra were run in $CDCl^3$ using the residual $CHCl_3$ protons as an internal reference. Carbon nuclear magnetic spectra (^{13}C NMR) were obtained using the Bruker AMX-300 instrument at 75.5 MHz in $CDCl_3$. All chemical shifts are given in ppm relative to tetramethylsilane.

Isolation and Identification. Sixteen frozen male castor sacs (970 g) were blended in a Waring Laboratory Blender for 2 min in methylene chloride (500 ml). The homogenate was filtered through a large Büchner funnel, and the solvent was removed by fractional distillation through a Vigreux column (17.5 cm) to prevent loss of the low boiling point fraction. The castoreum was treated with dilute sulfuric acid (200 ml of 2 N) and extracted with ether (3 × 200 ml) to isolate the neutral, acidic, and phenolic materials. The ether extract was washed with saturated sodium bicarbonate (3 × 130 ml) to remove the acidic compounds. The ether solution was then treated with enough sodium hydroxide to bring the pH to 12 (ca. 200 ml of 20%) in order to remove phenolic materials. The remaining ether solution was washed completely with brine, dried over anhydrous sodium sulfate, filtered, and the solvent removed by fractional distillation.

Synthetic Work: All compounds that were identified and not commercially available were synthesized except for nojigiko alcohol and junenol. Enough material was made not only to confirm our identification, but also for field bioassays with beaver.

6-Methyl-1-heptanol (2). 4-Methyl-1-pentanol (5.11 g, 50 mmol) and pyridine (0.5 g, 6 mmol) were dissolved in methylene chloride (8 ml), and freshly distilled phosphorus tribromide (13.5 g, 50 mmol) in methylene chloride (8 ml) was added dropwise over 30 min with stirring at −20°C under a nitrogen atmosphere (Scheme 1). After the addition was completed, the mixture was stirred for another hour at 0°C. The reaction mixture was treated with water



1 2

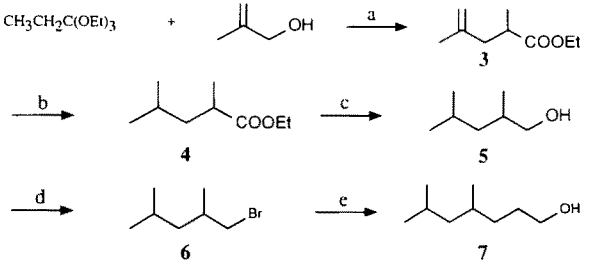
(a) PBr_3-pyridine, 1.5 hours, -20-0°C; (b) Mg, THF;
(c) ethylene oxide, -20°C; (d) HCl-H_2O.

SCHEME 1.

(5 ml), saturated sodium bicarbonate and brine, and dried over anhydrous sodium sulfate. The crude 1-bromo-4-methylpentane was purified by distillation (70.0–71.5°C/53 mm Hg) to yield **1** 2.30 g (28%).

Active magnesium powder (322 mg, 13.2 mmol) was placed in anhydrous THF (8 ml), and 1-bromo-4-methylpentane (2.20 g, 13.3 mmol) in anhydrous THF (16 ml) was slowly added. The reaction mixture was stirred for 4 hr at room temperature under nitrogen. Anhydrous cuprous iodide (250 mg, 1.3 mmol) was added after cooling to −10°C. Ethylene oxide (3 g, 68 mmol) was added through a tube with its end about 10 mm above the surface of the liquid (Dreger, 1941); the temperature was maintained at −20°C overnight. Water (3 ml) was added to the mixture, and the mixture was filtered through a Büchner funnel. The residue was rinsed with hexane (4 × 5 ml), and the combined organic phase was washed with dilute hydrochloric acid (3 N) and brine and dried over anhydrous sodium sulfate. The crude alcohol was purified by distillation (152–159°C) to yield 1.64 g (95%) of (2) as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 3.67–3.63 (t, 2H, CH_2OH), 2.61 (s, 1H, OH), 1.58–1.48 (m, 3H, CH, CH_2), 1.38–1.29 (m, 4H, CH_2CH_2), 1.27–1.13 (m, 2H, CH_2), 0.86 (d, 6H, $CHMe_2$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 63.0, 38.9, 32.7, 27.9, 27.1, 25.9, 22.6.

4,6-Dimethylheptanol (7). Unsaturated ester (**3**) was prepared by Claisen rearrangement (Johnson et al., 1970) (Scheme 2). 2-Methyl-2-propen-1-ol



(a) CH_3CH_2COOH, heat; (b) H_2, Pd-C; (c) $LiAlH_4$;
(d) TsCl; LiBr-acetone; (e) Mg, THF; ethylene oxide, -20°C.

SCHEME 2.

(5.95g, 83 mmol) was added to six equivalents ethyl orthopropionate containing a few drops of propionic acid. The ethanol produced in the reaction was continuously removed by fractional distillation. The crude ester was purified by distillation to yield 12.0 g (93%) of compound **3**. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 4.68 (d, 2H, $=CH_2$), 4.08 (q, 2H, OCH_2CH_3), 2.69–2.30 (m, 2H,

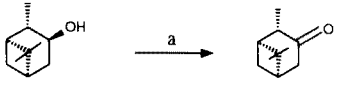
CH_2), 2.01 (q, 1H, CH), 1.67 (s, 3H, CH_3), 1.21 (t, 3H, OCH_2CH_3), 1.11 (d, 3H, CH_3). Catalytic hydrogenation (Augustine, 1965) of compound **3** in absolute ethanol over Pd-C at room temperature yielded 11.3 g (86%) of 2,4-dimethylvalerate **4**. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 4.08 (q, 2H, OCH_2CH_3), 2.49 (q, 2H, CH_2), 1.64–1.51 (m, 2H, 2CH), 1.21 (t, 3H, OCH_2CH_3), 1.17 (d, 3H, CH_3), 0.88 (d, 3H, CH_3), 0.87 (d, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 177.2, 60.0, 43.1, 37.7, 25.8, 22.3, 17.7, 14.3. The saturated ester **4** was added to 0.6 equivalent $LiAlH_4$ suspended in dry ether (Moffett, 1963). After stirring for 2 hr at room temperature, the mixture was quenched with H_2O–15% NaOH–H_2O. The precipitate was filtered and rinsed with ether. The combined organic solution was washed with brine and dried over anhydrous sodium sulfate. After removal of the solvent, the crude product was distilled to yield 7.37 g (89%) of 2,4-dimethyl-1-pentanol. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 3.51–3.44 (m, 2H, OCH_2), 1.74 (bs, 1H, OH), 1.75–1.56 (m, 1H, CH), 1.23–1.13 (m, 1H, CH), 1.04–0.95 (m, 2H, CH_2), 0.88 (d, 3H, CH_3), 0.87 (d, 6H, $2CH_3$). ^{13}C NMR (75.5 MHz, $CDCL_3$): δ (ppm) 68.6, 42.3, 33.5, 25.4, 23.9, 22.1, 17.0.

The alcohol (**5**) (7.37 g, 63 mmol) was added to 1.3 equivalent toluenesufonyl chloride in CH_2Cl_2 and pyridine at 0°C (Marvel and Sekera, 1955). The mixture was kept in a refrigerator overnight. The crystals were filtered and rinsed with hexane. The combined organic phase was washed with cold water, 2 N HCl, saturated $NaHCO_3$, and brine and dried over anhydrous sodium sulfate. After removal of solvent, the tosylate was added to three equivalents of LiBr in acetone and stirred overnight at room temperature (Wilt et al., 1970). After removal of the solvent, the syrup was extracted with hexane, and the organic phase was washed with water and brine, and dried over anhydrous sodium sulfate. After removal of the solvent, the crude product was purified by distillation to yield 10.1 g (94%), of 1-bromo-2,4-dimethylpentane (**6**) as a colorless liquid. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 3.41–3.24 (m, 2H, $BrCH_2$), 1.96–1.78 (m, 1H, CH), 1.73–1.54 (m, 1H, CH), 1.33–1.22 (m, 1H, CH_2), 1.15–1.01 (m, 1H, CH_2), 0.97 (d, 3H, CH_3), 0.88 (d, 3H, CH_3), 0.87 (d, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 44.3, 41.8, 32.9, 25.2, 23.0, 22.3, 18.8.

Active magnesium powder (312 mg, 12.8 mmol) was placed into anhydrous THF (7 ml), and 1-bromo-2,4-dimethylpentane (2.01 g, 11.2 mmol) in anhydrous THF (14 ml) was slowly added to it. The reaction mixture was stirred for 4 hr at room temperature under nitrogen. The solution was cooled to −10°C and anhydrous cuprous iodide (200 mg, 1.2 mmol) was added. Ethylene oxide (0.57 g, 13.3 mmol) was added through a tube whose end was about 10 mm above the surface of the liquid; the temperature was maintained at −20°C overnight. Water (3 ml) was added to the mixture, and the solution was filtered through a Büchner funnel. The residue was rinsed with hexane (4 × 5 ml), and

the combined organic phase was washed with dilute hydrochloric acid (3 N) and brine and dried over anhydrous sodium sulfate. The crude alcohol was purified by distillation (146–150°C) to yield 1.25 g (87%) of **7** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 3.63 (t, 2H, CH_2OH), 1.69–1.42 (m, 4H, 2CH, CH_2), 1.39–1.27 (m, 2H, CH_2), 1.20–1.07 (m, 2H, CH_2), 1.06–0.89 (s, 1H, OH), 0.89–0.83 (sextet, 9H, $3CH_3$). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 63.5, 46.7, 33.3, 30.3, 30.2, 25.3, 23.4, 22.3, 19.7.

Isopinocamphone (8). Pyridinium chlorochromate (18 g, 84 mmol was dissolved in methylene chloride (100 ml), and (1*S*, 2*S*, 3*S*, 5*R*)-(+)-isopinocampheol (4.63 g, 30 mmol) in methylene chloride (15 ml) was added at room temperature (Corey and Suggs, 1975). After the addition was completed, the reaction mixture was stirred for 2 hr and diluted with anhydrous ether (60 ml).



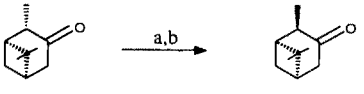
8

(a) PCC-CH_2Cl_2

SCHEME 3.

The solution was decanted, and the black residue was washed twice with ether. The combined organic solution was filtered through a short column packed with Florisil. The solvent was removed by rotary evaporator. The residual oil was purified by distillation (59.5–60.5°C/1.75 mm Hg) to yield 2.98 g (65%) of **8** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 2.56–2.46 (m, 2H, CHC=O, CH_2C=O), 2.37–2.28 (m, 2H, 2CH), 2.05–1.86 (m, CH_2), 1.19 (s, 3H, CH_3), 1.08 (d, 3H, CH_3), 0.76 (s, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 214, 51.2, 45.0, 44.8, 39.0, 34.4, 27.5, 21.8, 16.7.

Pinocamphone (9). Sodium (0.20 g, 8.7 mmol) was dissolved in absolute ethanol (5 ml), and the solution was cooled to 0°C; isopinocamphone (**8**) (1 g, 6.5 mmol) was then added quickly (Scheme 4). The reaction mixture was stirred overnight and neutralized with dilute hydrochloric acid (3 N). The crude product



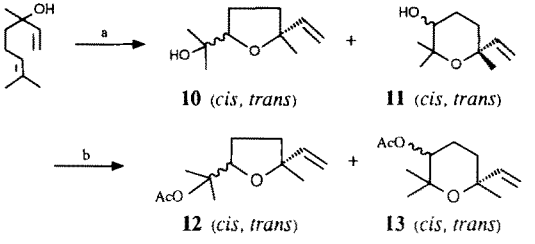
8 **9**

(a) NaOEt-EtOH; (b) H_2O-HCl

SCHEME 4.

(**9**) and starting material (**8**) were taken up in ether, and the organic phase was washed with brine and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by separation by flash chromatography (hexane–EtOAc, 10:1) yielded 0.39 g (40%) of **9** as a colorless oil. ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 2.63–2.57 (m, 2H, $CH_2C{=}O$), 2.45–2.29 (m, 3H, $CH_2C{=}O$, CH), 2.10–1.84 (m, 2H, CH_2), 1.28 (s, 3H, CH_3, H-9), 1.05 (d, 3H, CH_3, H-10), 0.84 (s, 3H, CH_3, H-9), ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 216, 51.2, 46.3, 44.3, 39.3, 38.8, 29.5, 26.4, 20.0, 15.5.

*Oxides of linalool (**10** and **11**) and their acetates (**12** and **13**).* Our synthesis (Scheme 5) was based on that of Klein et al. (1964) for the oxidation of linalool.



(a) MCPBA-CH_2Cl_2; (b) CH_3COCl-DMAP-pyridine-CH_2Cl_2.

SCHEME 5.

m-Chloroperbenzoic acid (MCPBA, 4.3 g, 25 mmol) was dissolved in CH_2Cl_2 (30 ml) and cooled to 0°C; linalool (3.1 g, 20 mmol) was added dropwise over 30 min with stirring at 0°C. After the addition was completed, the mixture was stirred for 5 hr at 0°C, then stirred overnight at room temperature. The mixture was filtered through a Büchner funnel, and the filtrate was treated with aqueous NaOH (10%, 3 × 10 ml), washed with brine (3 × 10 ml), and dried over anhydrous sodium sulfate. Evaporation of the solvent followed by flash chromatography (hexane–EtOAc, 10:1) yielded 1.61 g (47%) of **10** and 0.36 g (11%) of **11**. Separation of isomers of **10** by packed column gas chromatography (column D) yielded *cis*- and *trans*-isomers of linalool oxide. *trans*-Linalool oxide: ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 5.87–5.76 (four d, 1H, =CH), 5.16–5.09 (d t, 1H, $=CH_2$), 4.95–4.90 (d t, 1H, $=CH_2$), 3.76–3.70 (t, 1H, CHOH), 2.27 (s, 1H, OH), 1.88–1.61 (m, 4H, CH_2), 1.25 (s, 3H, CH_3), 1.16 (s, 3H, CH_3), 1.07 (s, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 143.7, 112.0, 86.1, 83.0, 72.0, 37.7, 27.0, 26.3, 25.8, 24.1. *cis*-Linalool oxide: ^{1}H NMR (300 MHz, $CDCl_3$): δ (ppm) 5.99–5.89 (q, 1H, =CH), 5.19–5.12 (d d, 1H, $=CH_2$), 5.01–4.95 (d d, 1H, $=CH_2$), 3.83 (t, 1H, CHOH), 2.10 (s, 1H, OH), 1.92–1.71 (m, 4H, CH_2), 1.28 (s, 3H, CH_3), 1.20 (s, 3H,

CH_3), 1.10 (s, 3H, CH_3). ^{13}C NMR (75.5 MHz, $CDCl_3$): δ (ppm) 144.9, 112.0, 85.6, 82.8, 71.9, 37.7, 27.3, 26.4, 25.9, 24.2. Mixture **11** was not separated but was directly converted to its acetate.

Freshly distilled acetyl chloride (117.8 mg, 1.50 mmol) was added to pyridine (240 mg, 3 mmol) and 4-dimethylaminopyridine (DMAP, 5 mg, 0.04 mmol) in 1.5 ml CH_2Cl_2. The solution was cooled to −20°C and oxide **10** or **11** (120 mg, 0.71 mmol in 0.5 ml CH_2Cl_2) was added. The reaction mixture was kept at 0°C for 3 hr and at room temperature overnight. The mixture was cooled to −20°C, the supernatant decanted, and the precipitate washed with 0.5 ml CH_2Cl_2. The combined organic phases were treated with water (1 ml), hydrochloric acid (3 N 2 × 0.5 ml), and brine (2 × 0.5 ml) and dried over anhydrous sodium sulfate. The crude ester was purified by gas chromatography (packed column D) to yield 80 mg (53%) of acetate **12** and **13**. ^1H NMR of **12**: δ (ppm) 4.11–4.03 [q, 1H, −O−CH−$C(Me)_2$−OAc]. ^1H NMR of **13**: δ (ppm) 4.62–4.57 [q, 1H, −O−$C(Me)_2$−CHOAc].

RESULTS

Our reinvestigation of the neutral compounds in beaver castoreum led us to identify the 16 neutral compounds listed in Tables 1 and 2. Of these 16 compounds, only three had previously been reported as constituents of castoreum. Table 1 lists the characterization methods and structures of the oxygen-containing monoterpenes. Of these nine monoterpenes, only borneol was previously known as a constituent of castoreum (Walbaum and Rosenthal, 1927). Table 2 lists the characterization methods and structures of other neutral compounds. Among these seven compounds, acetophenone and benzyl alcohol had been reported as constituents of castoreum before (Walbaum and Rosenthal, 1927; Lederer, 1946, 1949). The remaining five—two aromatic compounds, two branched aliphatic alcohols, and a sesquiterpene—were found in castoreum for the first time.

Table 3 gives the average amounts (in milligrams) of the various neutral compounds found in one beaver castor sacs (gland), termed one gland equivalent (GE). The quantity refers to the amount of each individual neutral compound per castor gland. The determination of GE is as follows: The most abundant neutral compound, borneol, was isolated and weighed directly. The gland equivalents of the remaining neutral compounds were obtained by comparing the GC peak areas of each compound to that of borneol.

Of the nine neutral compounds reported by Lederer as being constituents of castoreum, only three were found in this study: benzyl alcohol, acetophenone, and borneol. The other six neutral compounds reported by Lederer, but not found in our neutral fractions are cholesterol, β-cholestanol, mannitol, choles-

TABLE 1. NEUTRAL COMPOUNDS IN CASTOREUM OF BEAVER (*Castor canadensis*): OXYGEN-CONTAINING MONOTERPENES

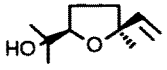
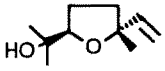
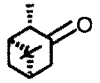
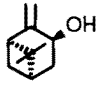
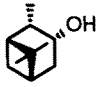
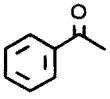
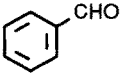
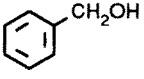
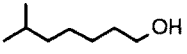
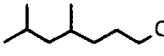
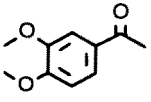
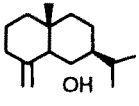
Name	Structure	Co-injection		Isolated and confirmed by						
				GC-MS		NMR				
		DB-1	FFAP	CI	EI	^{1}H	^{13}C	COSY	Hetcor	Synthesis
cis-Linalool oxide		X	X	X	X	X	X			X
trans-Linalool oxide		X	X							X
Isopinocamphone		X	X	X	X	X	X			X
trans-Pinocarveol		X	X	X	X	X	X			
Pinocamphone		X	X	X	X	X	X			X
Nojigiko alcohol				X	X	X	X	X	X	
Verbenone		X	X	X	X	X	X		X	
(1*R*)-Myrtenol		X	X	X	X	X	X			
Borneol		X	X	X	X	X	X			

TABLE 2. NEUTRAL COMPOUNDS IN CASTOREUM OF BEAVER (*Castor canadensis*): OTHERS

Name	Structure	Coinjection		Isolated and confirmed by						
				GC-MS		NMR				
		DB-1	FFAP	CI	EI	^{1}H	^{13}C	COSY	Hetcor	Synthesis
Acetophenone		X		X	X	X	X			
Benzaldehyde		X	X	X	X	X				
Benzyl alcohol		X	X	X	X	X				
6-Methyl 1-heptanol		X	X	X	X	X	X			X
4,6-Dimethyl 1-Heptanol		X		X	X	X	X			X
3,4-Dimethoxy Acetophenone		X	X	X	X	X	X			
Junenol				X	X	X	X	X	X	

terol oleate, *cis*-5-hydroxytetrahydronol, and 4-methoxyacetophenone. Except for 4-methoxyacetophenone, these compounds may not be volatile enough to be detected by our methods. The other possibility is that Lederer appears to have used a different species of beaver (*Castor fiber*), but his papers are not clear on this point.

Eight compounds—6-methyl-1-heptanol, 4,6-dimethyl-1-heptanol, iso-

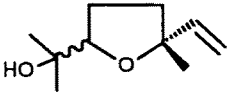
TABLE 3. AVERAGE AMOUNTS OF NEUTRAL COMPOUNDS FOUND IN ONE BEAVER CASTOR SAC

Name	Gland equivalent (mg)
Acetophenone	0.18
Benzaldehyde	0.65
Benzyl alcohol	2.01
6-Methyl-1 heptanol	0.35
4,6-Dimethyl-1-heptanol	0.53
3,4-Dimethoxyacetophenone	1.20
Junenol	1.03
trans-Linalool oxide	0.45
cis-Linalool oxide	0.46
Isopinocamphone	0.35
trans-Pinocarveol	1.52
Pinocamphone	0.42
Nojigiko alcohol	1.19
Verbenone	3.33
(1*R*)-Myrtenol	0.29
Borneol	16.9

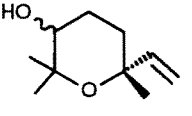
pinocamphone, pinocamphone, and two linalool oxides and their acetates—were synthesized as described under Methods and Materials.

DISCUSSION

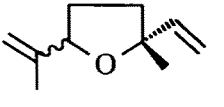
Many of the neutral castoreum constituents occur in plants or in excretions of mammals. Linalool oxides have been isolated from *Cryptocaria aschersoniana* oil of southern Brazil (Naves et al., 1963). Even though they have been used as flavor and perfume agents for some time, the first definite structure was not established until 1963 (Felix et al., 1963). They first oxidized (±)-linalool with monoperoxyphthalic acid yielding two pairs of diastereometric oxides, $C_{10}H_{18}O_2$ (**9**: *cis*, *trans* and **10**: *cis*, *trans*).



9 (*cis*, *trans*)



10 (*cis*, *trans*)



13 (*cis*, *trans*)

Furans **9** are the major products. The structures of **9** (*cis*, *trans*) were deduced by conversion of **9** to its benzoate, which was pyrolyzed at 180–220°C to give a mixture of two doubly unsaturated ethers (**13**). IR analysis showed the presence of a vinyl group and a methylene double bond; ^{1}H NMR analysis gives a multiplet at δ (ppm) 4.2–4.3, which is a proton in the environment of C=C−CH−O. Therefore, Felix et al. named these tetrahydrofuran derivatives "linalool oxide," which was accepted by the *Handbook of Terpenoids* (Dev et al., 1982) and many others. However, there are some differences in the ^{1}H NMR data of our isolated compounds with those reported by Felix et al. This may be due to different instruments or solvent effects. Pure **9** (*cis*, *trans*) and **10** (*cis*, *trans*) are not commercially available. To determine whether our isolated compounds are furan-type or pyran-type compounds, a modified method was employed to convert linalool into its oxides, which were separated into two mixtures, **9** and **10**, by flash chromatography. These two mixtures, **9** and **10**, were converted to acetates **11** and **12**, respectively. Comparing the ^{1}H NMR data of the alcohols and their esters, the peak at δ (ppm) 3.83–3.78 (t) in alcohol **9** shifts to δ (ppm) 4.10–4.03 (q) in ester **11**, while the peak at δ (ppm) 3.44–3.38 (t) in alcohol **10** shifts to δ (ppm) 4.62–4.54 (q) in ester **12**. The former small difference is caused by a field effect of the OAc group; the latter big difference is caused by a scalar effect of the OAc group (Duddeck and Dietrich, 1989). In summary, this experiment gives another proof that **9** (*cis*, *trans*) are tetrahydrofuran derivatives, and **10** (*cis*, *trans*) are tetrahydropyran derivatives.

A further separation of **9** (*cis*, *trans*) on column D gave pure *cis*- and *trans*-linalool oxides, whose ^{1}H NMR data and ^{13}C NMR data coincide with our isolated compounds. In the *cis*-isomer, the proton attached to the C=C was deshielded by the *cis*-OH group and its chemical shift is further downfield than that of the *trans*-isomer.

Isopinocamphone is a major constituent of the essential oil from the mint, *Mentha aquatica* L. found in southern Europe (Shimizu et al., 1966). The compound was prepared from isopinocampheol by oxidation with CrO_3 (Zweifel and Brown, 1964). In our method, we employed the more effective reagent pyridium chlorochromate. The chemical shifts of three characterizing methyl groups (C_8, C_9, and C_{10}) (Erskin and Knight, 1960) are in good agreement with our isolated and synthetic samples.

(−)-Pinocamphone can constitute up to 45% of the oil of *Hyssopus officinialis* L. (Guenther, 1949). Pinocamphone was synthesized from α-pinene by treatment with perphthalic acid and acidic alumina (Nigam and Levi, 1968). The epimerization of isopinocamphone to pinocamphone was employed in our work (Zweifel and Brown, 1964). The equilibrium favors pinocamphone. The reported ^{1}H NMR spectrum (Erskin and Knight, 1960) is identical with our isolated and synthetic compounds.

trans-Pinocarveol has been found in Spanish eucalyptus, *E. globulus* (Schmidt, 1944). The ^{1}H NMR spectrum of the synthetic compound (Jashi et al., 1968; Heikman et al., 1968) is the same as that from our isolated fraction.

Nojigiku alcohol and its acetate are major components of the essential oil of *Chrysanthemum japonense* (Uchio, 1977). The ^{1}H NMR spectrum (Uchio, 1977) is identical with our isolated fraction, and the ^{13}C NMR data (Darby et al., 1978) agree with our sample.

Verbenone (**14**) occurs in small amounts in *Verbena* and various other species (Kerschbaum, 1900). The ^{1}H NMR spectrum of synthetic verbenone (Bates and Thalacker, 1988) is identical with that of our isolated sample.

Both (+)- and (−)-myrtenol have been isolated from the essential oil of *Cyperus africalatus* L. in West Africa by vacuum fractionation (Couchman et al., 1964). The ^{1}H NMR of myrtenol (Heikman et al., 1968) is nearly the same as that from our isolated fraction. The only difference is that the peak of CH_2OH is reported at 3.89 ppm (a mutiplet in Heikman's paper), and in our spectrum it is at 3.98 ppm, doublet (J = 1.7 Hz). This split is caused by long-range olefinic proton coupling.

No natural source of the two branched alcohols was found in the literature. The first synthesis of 6-methyl-1-heptanol was reported by Levene and Allen (1916). They treated 4-methylpentyl iodide with malonic ester and reduced the resulting ester with Na-EtOH to give the above alcohol. This method requires more steps than ours. The ^{1}H NMR and ^{13}C NMR data of the synthesized 6-methyl-1-heptanol agree with authetic data (Sadtlar, 1980, 1981). The synthesis of 4,6-dimethyl-1-heptanol has also been reported (S. Julia, et al., 1961, M. Julia, et al., 1966). In Julia's work (1961), the starting material, 1,1-diethoxy-1-cyclopropylethane, was converted to 1,3-dimethyl 1-cyclopropyl-1-butanol in six steps. The homoallylic rearrangement was accomplished by treatment of the alcohol with HBr. The resulting unsaturated bromide was converted to 4,6-dimethyl-1-heptanol by catalytic hydrogenation. In our work, the starting material, 2,4-dimethylpentyl bromide, was prepared from triethyl orthopropionate and 2-methylallyl alcohol via Claisen rearrangement and three subsequent steps. Our synthetic route is short and the yield is good.

Junenol is an eudesmane class sesquiterpene. A dextrorotatory alcohol, junenol was first isolated from the essential oil of juniper berries (Herout et al., 1954). The first total synthesis of (±)-junenol was completed by Schwarz et al. (1972). From our isolated fraction, we acquired ^{1}H NMR, ^{13}C NMR, HETCOR, and HH COSY (Duddeck and Dietrich, 1989). From the NMR data, the structure of our isolated compound was deduced as junenol. The ^{1}H NMR of the isolated compound [(300 MHz, CDC_3): δ (ppm) 4.96 (d, 1H), 4.66 (d, 1H), 3.65 (t, 1H), 2.35–1.25 (m, 14H), 0.96 (d, 3H), 0.87 (s, 3H), 0.73 (s, 3H)] agrees with Schwartz's data (Schwartz et al., 1972). The ^{13}C NMR data are not

available in the literature. The ^{13}C NMR (75.7 MHz, $CDCl_3$) of our sample: δ (ppm) 148.3, 106.3, 67.2, 58.3, 49.8, 41.9, 40.4, 37.9, 37.6, 26.8, 24.0, 21.1, 18.4, 17.5, 16.1.

A number of the described compounds are known as detoxification products in the urine of various herbivores and also of carnivores. For instance, in rabbits *trans*-pinocarveol is derived from β-pinene and myrtenol is a metabolite of α-pinene. In rabbits and brushtail possums, verbenol is formed from α-pinene. Acetophenone occurs in the urine of dogs and cats that have been fed cinnamic acid. Benzyl alcohol is excreted by dogs and cats that had been given benzyl acetate. In rabbits, borneol is a metabolite of camphor. Benzaldehyde was found in rat hepatocytes as a metabolite of benzylamine. These numerous studies are summarized by Scheline (1991). The beaver appears to sequester metabolites of secondary plant compounds in its castor sac.

Our purpose for identifying the neutral constituents from beaver castoreum was to determine what role these and other compounds, such as phenolics and basics from beaver scent marks, play in chemical communication. Beaver possibly use at least some of the neutral compounds for intraspecific communication via their scent marks. Several studies have compared beaver responses to whole castoreum and to its components. The neutral fraction released responses from beaver in experiments by Svendsen and Huntsman (1988). While some of the phenolic castoreum constituents have been shown to elicit responses from beaver (Müller-Schwarze and Houlihan, 1991, Schulte et al., 1994), only one neutral compound, borneol, has been bioassayed individually so far (Schulte et al., 1994). A mixture of 12 neutrals reported here released slight to moderate responses from beaver. The 12 tested neutral compounds were borneol, benzaldehyde, benzyl alcohol, 6-methyl-1-heptanol, *cis*- and *trans*-linalool oxide, 4,6-dimethylheptane-1-ol, *trans*-pinocarveol, isopinocamphone, verbenone, (1*R*)-myrtenol, and 3,4-dimethoxyacetophenone. A fivefold higher concentration of the same mixture rendered it more active. Finally, a mixture of these 12 neutrals and 14 phenolics proved to be almost as active as whole castoreum (Schulte et al., 1994). The beaver appear to recycle catabolites and detoxification products by storing them in their castor sacs for scent marking when needed. This added benefit may offset the cost of detoxification.

Having identified a series of neutral compounds, we can now analyze individual scent mounds and castoreum from specific individuals on a routine basis. The chemical identifications reported here have opened the door for selecting and manipulating specific compounds in castoreum. In our experiments, borneol released scent-marking responses (Schulte et al. 1994). Castoreum composition profiles may be indicative of individuals, sex, age, colony membership, diet, population, or species. Knowledge of the precise pattern of compounds will permit analysis of genotypic and environmental influences on castoreum composition.

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