formulation (TF1169) is a racemic mixture of the enantiomeric pair of isomers designated at *R,S* or (±)-fluazifop-butyl. On the other hand, TF1195 is formulated at 125 g L^{-1} since it contains only the *R* or + enantiomer (active isomer) of fluazifop-butyl.

Soybean samples were analyzed separately as plant and seeds. Soybean plant or seed samples field-treated with Fusilade showed no detectable fluazifop-butyl residues greater than 0.05 $\mu g\ g^{-1}$. The soybeans analyzed were treated at two rates (1.0 and 2.0 L ha^{-1}), with the period between herbicide application and harvest being greater than 90 days. Although no residues were detected in beans, it may be of future interest to examine soy oil for fluazifop-butyl residues.

The results of the analyses on potato samples were more remarkable, although the recovery data given in Table II suggested better recoveries when a 25-g sample was used rather than a 50-g sample. The method was verified for a 50-g sample in each substrate and various fortification levels. Potatoes analyzed were obtained from two separate trials, each being treated with Fusilade, with the two different formulations, i.e., TF1169 and TF1195, being used in these studies. Potatoes treated at two rates (1.0 and 2.0 L ha^{-1}) postemergence of TF1169 (250 g L^{-1}) showed no detectable residue (<0.01 $\mu g\ g^{-1}$) when harvested 90 days after application. On the other hand, potatoes treated two rates of TF1195 (125 g L^{-1}) showed residues of 0.16 and 0.47 $\mu g\ g^{-1}$ (fluazifop-methyl ester) for the 1.0 and 2.0 L ha^{-1} rates, respectively. It must be noted that these potatoes were sampled only 42 days after the application, which is considerably shorter than the label recommendation of 90 days prior to harvest.

Currently, this method is being examined for its suitability in determining fluazifop-butyl residues in other crops and vegetables. The method shows potential as a multiresidue method for determination of the phenoxyphenoxy class of herbicides, and efforts are being made to assess its applicability in the analysis of other similar herbicides such as flamprop-methyl, fenoxaprop-ethyl, fenthiaprop-ethyl, sulfometuron, and haloxyfop-methyl.

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Registry No. Fluazifop-butyl, 69806-50-4.

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The Intensely Sweet Sesquiterpene Hernandulcin: Isolation, Synthesis, Characterization, and Preliminary Safety Evaluation

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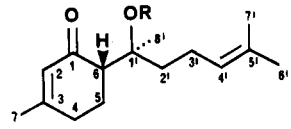
Details are provided of the isolation of an intensely sweet compound, hernandulcin, from *Lippia dulcis* Trev. (Verbenaceae), in addition to its synthesis, stereochemical assignation, spectroscopic and chromatographic characterization, derivatization, and thermal stability. This compound, as well as its analogue, epihernandulcin, and its products of thermal degradation, 3-methyl-2-cyclohexen-1-one and 6-methyl-5-hepten-2-one, exhibited LD_{50} values in excess of 1 g/kg of body weight, when tested for acute toxicity in mice. All four compounds were also found to be nonmutagenic in forward mutation assays utilizing *Salmonella typhimurium* strain TM677.

In a preliminary report (Compadre et al., 1985), we described the occurrence of the intensely sweet compound hernandulcin (1), a constituent of the herb *Lippia dulcis*



1 R = H
7 R = Ac

Trev. (Verbenaceae), grown in Mexico. The racemic form of hernandulcin has been efficiently synthesized by a di-

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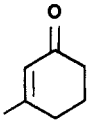
rected aldol condensation, using 3-methyl-2-cyclohexen-1-one (2) and 6-methyl-5-hepten-2-one (3) as starting



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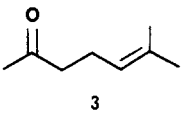

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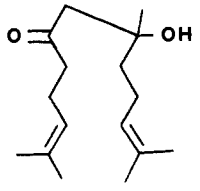
materials. This bisabolane sesquiterpene is the prototype of a new class of intense sweeteners, and, after being established as nonmutagenic and nontoxic for mice after single-dose oral administration, natural (+)-hernandulcin was rated at 1000 times sweeter than sucrose on a molar basis by a human taste panel (Compadre et al., 1985). Recently, Mori and Kato (1986) suggested that the absolute configuration of the natural form of hernandulcin is 6S,1'S, based on its synthesis from (R)-(+)-limonene and its previously reported relative configuration (Compadre et al., 1985). An account of the past and present uses of *L. dulcis*, and the analysis of the volatile oil constituents of this species that cooccur with hernandulcin, has been published elsewhere (Compadre et al., 1986).

In this paper, we detail the isolation of hernandulcin from *L. dulcis* and elaborate on its chromatographic and spectroscopic characteristics. In addition, the identification of three minor reaction products (4–6) obtained in the



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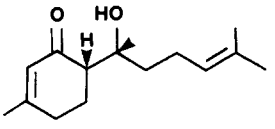

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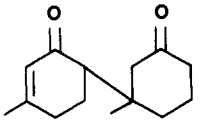

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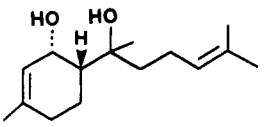
synthesis of (±)-hernandulcin will be reported. When heated at the relatively high temperature of 140 °C, hernandulcin has been found to undergo a reversed aldol condensation to regenerate its synthetic precursors 2 and 3, a pattern consistent with other β-ketols (House et al., 1973; Mukaiyama, 1982). Since small amounts of 2 and 3 could conceivably be produced from hernandulcin on long-term storage at lower temperatures, their mutagenic and acute toxicity potentials have been evaluated in this investigation.

It is currently accepted that a molecular feature common to most compounds that exhibit a sweet taste is the presence of an AH,B system, where A and B are electronegative atoms, H is a covalently bonded proton, and the interatomic distance between A and B is about 3 Å (Shallenberger et al., 1969; Crosby et al., 1979). We have pointed to the hernandulcin C-1' hydroxyl and C-1 carbonyl groups as the possible AH,B system responsible for sweetness, since their chemical modification resulted in nonsweet derivatives (Compadre et al., 1985). In the present study, spectral data are reported for the acetylated (7) and hydride reduction (8) products of hernandulcin, which do not exhibit a sweet taste.

EXPERIMENTAL SECTION

Optical rotations were determined on a Perkin-Elmer 241 polarimeter. UV and IR spectra were recorded with a Cary 118 spectrophotometer (run in MeOH) and a Nicolet MX-1 FT-IR spectrophotometer, respectively. ^1H and ^{13}C NMR spectra were recorded with either a Nicolet NT-360 or a Varian XL-300 spectrometer, with Me_4Si as the internal standard. Low-resolution and high-resolution mass spectra were obtained, respectively, on Varian MAT 112S and Varian 731 instruments, both of which were operated at 70 eV. Column chromatography was conducted with silica gel, particle size 63–200 μm (E. Merck, Darmstadt, W. Germany), while low-pressure column chromatography involved the use of a Lobar column (size A), prepacked with Lichoprep Si 60, particle size 40–63 μm (Merck). Analytical and preparative TLC were performed on Merck silica gel G plates, with 250-μm-thick layers, which were visualized either by examination under short-wave UV light or after spraying with 60% w/v H_2SO_4 and heating at 110 °C for 10 min. HPLC analyses were carried out with a Beckman Model 321 gradient liquid chromatograph connected to a Model 421 controller, a Model 100A pump, and a Model 210 sample injector. HPLC detection was achieved by means of a Perkin-Elmer LC-85 variable-wavelength UV detector with LC autocontrol. GC/MS was conducted on a Finnigan 4510 mass spectrometer, equipped with INCOS software. The GC/MS interface was a deactivated vitreous silica capillary direct line. The instrument was operated over the mass range 43–280, with an emission current of 0.25 mA and a scan rate of 1 scan/s.



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Plant Material. Samples of *L. dulcis* Trev. (Verbenaceae) herb were collected from a natural habitat in Tlayacapan, Morelos, approximately 60 km south of Mexico City, Mexico. Additional quantities of dried plant material were purchased from a medicinal plants market in Mexico City. Vouchers representing these collections (*Compadre 103–105*) have been deposited at the Field Museum of Natural History, Chicago, Il.

Isolation of (+)-Hernandulcin (1). Previous phytochemical studies performed on *L. dulcis* herb have suggested that its sweet principle is a substance of low polarity (Brodie and Jungk, 1881; Rio de la Loza, 1892; Breitwieser, 1943). Thus, in the present isolation work, air-dried chopped leaves and flowers of *L. dulcis* (900 g) were extracted with petroleum ether (bp range 60–80 °C, 4 × 4 L) at room temperature. The combined extracts were concentrated at reduced pressure to produce a sweet-tasting waxy yellow residue (7.35 g). A portion (ca. 1 mg) of this residue was dissolved in chloroform–methanol (1:1) and streaked on an analytical TLC plate that was developed with hexane–acetone (17:3). The TLC plate was dried at room temperature and examined under short-wave UV light, and the positions of a number of fluorescence-quenching zones were marked. A small sample (<0.1 mg) of a zone with R_f 0.50 was briefly tasted and expectorated and found to possess a distinctly sweet taste. In an initial isolation experiment, 1.4 g of the dried *L. dulcis* petroleum ether extract was chromatographed over silica gel by elution with hexane–acetone (9:1). Fractions were monitored by TLC on silica gel G by comparison with the sweet zone identified earlier, and the crude sweet component was purified by preparative TLC using hexane–acetone (17:3) to afford pure (6S,1'S)-hernandulcin (1; 7 mg, 0.004% w/w) as a colorless oil, $[\alpha]^{25}_D$ +109° (*c* 0.11, EtOH). Additional quantities of plant material were worked up in a similar manner to generate larger amounts of this sweet substance.

Table I. ^{13}C NMR Data for Hernandulcin (1) and Three Derivatives[a]

carbon	1[b]	5[b]	7[c]	8[b]
C-1	204.0 s	203.6 s	198.5 s	65.9 d
C-2	127.4 d	127.4 d	127.8 d	122.8 d
C-3	163.6 s	163.6 s	160.5 s	140.7 s
C-4	31.2 t	31.6 t	31.4 t	31.6 t
C-5	25.0 t	22.1 t	24.2 t	17.4 t
C-6	52.0 d	55.4 d	51.8 d	44.7 d
C-7	24.1 q	24.2 q	23.8 q	24.6 q
C-1′	73.9 s	74.4 s	85.4 s	74.3 s
C-2′	40.1 t	36.9 t	35.4 t	41.5 t
C-3′	21.5 t	22.1 t	22.0 t	23.1 t
C-4′	124.4 d	124.7 d	124.1 d	124.5 d
C-5′	131.4 s	131.5 s	131.7 s	131.4 s
C-6′	25.7 q	25.8 q	25.7 q	25.7 q
C-7′	17.6 q	17.7 q	17.6 q	17.7 q
C-8′	23.6 q	25.5 q	23.0 q	23.5 q
C=O (Ac)			170.6 s	
CH_3 (Ac)			22.3 q	

[a] Spectra were run in $CDCl_3$. Values are recorded in parts per million relative to Me_4Si. Multiplicity is designated as follows: s, singlet; d, doublet; t, triplet; q, quartet. [b] Spectrum obtained at 90.8 MHz. [c] Spectrum obtained at 75.4 MHz.

(+)-Hernandulcin (1) exhibited the following data: UV, λ_{max} 236 nm (log ϵ 4.23); IR, ν_{max} (film) 3365, 2970, 2919, 1644, 1381, 1215, 1194 cm^{-1}; ^{13}C NMR (90.8 MHz), see Table I; HR-MS, m/z (relative intensity) 236.18005 (M^+, $C_{15}H_{24}O_2$, 7%), 221.15489 ($C_{14}H_{21}O_2$, 5), 218.16887 ($C_{15}H_{22}O$, 14), 153.09318 ($C_9H_{13}O_2$, 19), 137.09823 ($C_9H_{13}O$, 11), 110.07485 ($C_7H_{10}O$, 100), 109.06727 (C_7H_9O, 96), 95.04983 (C_6H_7O, 40), 82.04153 (C_5H_6O, 21). The ^1H NMR spectroscopic parameters of 1, as well as a two-dimensional ^1H–^{13}C shift-correlated spectrum of this compound, have been presented previously (Compardre et al., 1985).

Synthesis of (±)-Hernandulcin (1). 3-Methyl-2-cyclohexen-1-one (2; 0.034 mol, 3.74 g) was added to a solution of lithium diisopropylamide (0.034 mol, 3.68 g) in dry tetrahydrofuran (35 mL) at −15 °C under nitrogen. After the mixture was stirred for 15 min, 0.034 mol (4.28 g) of 6-methyl-5-hepten-2-one (3) was incorporated, and stirring was continued an additional 10 min. The reaction was quenched by the addition of 10% NH_4Cl (100 mL), followed by extraction of the products into diethyl ether (3 × 50 mL). The combined organic layer was washed with saturated NaCl solution and dried over anhydrous Na_2SO_4. After filtration and removal of the solvent, 7.6 g of a yellow liquid was obtained and was purified by low-pressure column chromatography by elution with hexane–acetone (97:3) to afford, in turn, (±)-hernandulcin (1; 0.0164 mol, 3.88 g, 47.1%), as well as three minor reaction products, (±)-2,8,12-trimethyl-8-hydroxytrideca-2,11-dien-6-one (4; 0.028 g, 0.32%), (±)-epihernandulcin (5; 0.189 g, 2.35%), and (±)-6-(1-methyl-3-oxocyclohexyl)-3-methyl-2-cyclohexen-1-one (6; 0.051 g, 0.68%). Also obtained were unreacted quantities of the starting materials, 2 and 3. (±)-Hernandulcin, which represents a 1:1 mixture of the (6*S*,1′*S*) and (6*R*,1′*R*) enantiomers of this compound, was indistinguishable spectroscopically and chromatographically from the naturally occurring (+) form.

Characterization of Minor Reaction Products. (±)-2,8,12-Trimethyl-8-hydroxytrideca-2,11-dien-6-one (4) exhibited the following properties: colorless oil; UV, λ_{max} 212 nm (log ϵ 3.61); IR, ν_{max} 3503, 2922, 1700, 1450, 1376, 1109 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.18 (3 H, s, CH_3-15), 1.50 (2 H, m, H_2-9), 1.59 (6 H, br s, CH_3-14, -16), 1.65 (6 H, br s, CH_3-1, -13), 2.01 (2 H, m, H_2-10), 2.25 (2 H, m, H_2-4), 2.45 (2 H, t, J = 7.3 Hz, H_2-5), 2.51, 2.60 (2 H, AB system, J_{AB} = 17.1 Hz, H_2-7), 3.86 (1 H, br s, exchangeable with D_2O, OH-8), 5.05 (2 H, m, H-3 and H-11); ^{13}C NMR (90.8 MHz, $CDCl_3$) δ 17.5 (q, C-14, C-16), 22.0, 22.5 (two t, C-4, C-10), 25.2 (q, C-1, C-13), 26.6 (q, C-15), 41.8 (t, C-9), 44.4 (t, C-5), 51.4 (t, C-7), 71.4 (s, C-8), 122.2 (d, C-3), 124.0 (d, C-11), 131.5 (s, C-12), 132.8 (s, C-2), 212.9 (s, C-6); MS, m/z (relative intensity) 252 (M^+, 0.4%), 234 ($M - H_2O$, 15), 219 (5), 191 (4), 123 (10), 111 (15), 109 (29), 83 (32), 82 (24), 69 (100); mass measurement, found 234.19860, calcd for $C_{16}H_{26}O$, 234.19822.

(±)-Epihernandulcin (5) was obtained as a colorless oil and represents a 1:1 mixture of the (6*S*,1′*R*) and (6*R*,1′*S*) isomers of 1. This compound exhibited the following spectroscopic data: UV, λ_{max} 237 nm (log ϵ 4.18); IR, ν_{max} (film) 3375, 2971, 2919, 1644, 1381, 1215, 1194 cm^{-1}; ^{13}C NMR (90.8 MHz), see Table I; MS, m/z (relative intensity) 236 (M^+, 7%), 218 (13), 153 (20), 137 (11), 110 (100), 109 (91), 95 (45), 82 (26), 69 (35), 55 (30); mass measurement, found 236.17770, calcd for $C_{15}H_{24}O_2$, 236.17763. The ^1H NMR spectral characteristics of epihernandulcin (5) have appeared in a previous publication (Compadre et al., 1985).

(±)-6-(1-Methyl-3-oxocyclohexyl)-3-methyl-2-cyclohexen-1-one (6) was obtained as a yellowish gum: UV, λ_{max} 237 nm (log ϵ 4.16); IR ν_{max} (film) 2965, 2958, 2876, 1708, 1658 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 0.97 (3 H, s, CH_3-7′), 1.93 (3 H, s, CH_3-7), 2.10, 2.87 (2 H, AB system, J_{AB} = 13.6 Hz, H_2-2′), 5.81 (1 H, br s, H-2); ^{13}C NMR (90.8 MHz, $CDCl_3$) δ 21.8, 23.5 (two t, C-5, C-5′), 22.4 (q, C-7′), 23.6 (q, C-7), 31.3 (t, C-4), 33.0 (t, C-6′), 40.6 (s, C-1′), 41.0 (t, C-4′), 52.5 (t, C-2′), 52.6 (d, C-6), 128.0 (d, C-2), 160.2 (s, C-3), 200.1 (s, C-1′), 212.8 (s, C-1); MS, m/z (relative intensity) 220 (M^+, 4%), 205 (1), 123 (3), 110 (100), 95 (84), 82 (66), 67 (14), 55 (38), 41 (44); mass measurement, found, 220.14615, calcd for $C_{14}H_{20}O_2$, 220.14620.

Acetylation of 1. (±)-Hernandulcin (1; 0.5 mmol, 118 mg), in 0.4 mL of triethylamine, was treated with 0.4 mL of acetic anhydride in the presence of 4-(dimethylamino)pyridine (1.23 mmol, 150 g). After stirring at room temperature for 36 h, workup in the usual manner, and purification by preparative TLC, 67 mg (48%) of the colorless oil (7) was obtained. This compound exhibited the following spectral data: UV, λ_{max} 235 nm (log ϵ 4.32); IR, ν_{max} (film) 2930, 1732, 1669, 1376, 1257, 1109 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.62 (3 H, s, CH_3-7′), 1.67 (3 H, s, CH_3-6′), 1.71 (3 H, s, CH_3-8′), 1.92 (3 H, s, CH_3-7), 2.02 (3 H, s, $-OCOCH_3$), 2.31 (2 H, m, H_2-4), 3.48 (1 H, dd, J = 17.1, 4.6 Hz, H-6), 5.09 (1 H, br t, J = 6.1 Hz, 4′-H), 5.79 (1 H, br s, 2-H); ^{13}C NMR (75.4 MHz), see Table I; MS, m/z (relative intensity) 278 (M^+, 0.4%), 236 (1), 218 (34), 150 (17), 109 (100), 82 (25), 43 (70).

Reduction of 1. This reaction was carried out by the procedure described by Luche (1978) for the 1,2-reduction of α,β-unsaturated carbonyl compounds. (±)-Hernandulcin (1; 0.85 mmol, 200 mg) was treated with an equimolar amount of sodium borohydride in the presence of samarium chloride hexahydrate (0.85 mmol) in MeOH (0.5 mL). On workup and purification by preparative TLC in petroleum ether–acetone (17:3), 8 (80 mg, 40.0%) was obtained as a colorless oil: UV, λ_{max} 210 nm (log ϵ 3.69); IR, ν_{max} (film) 3365, 2966, 2914, 1450, 1439, 1414, 1377, 1289 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ 1.21 (3 H, s, CH_3-8′), 1.64 (3 H, s, CH_3-7′), 1.69 (3 H, s, CH_3-6′), 1.71 (3 H, s, CH_3-7), 4.42 (1 H, br s, $W_{h/2}$ = 10 Hz, H-1), 5.14 (1 H, m, H-4′), 5.59 (1 H, m, H-2); ^{13}C NMR (90.8 MHz), see Table I; MS, m/z (relative intensity) 238 (M^+, 0.3%), 220 ($M^+ - H_2O$, 2), 202 (3), 167 (34), 149 (100), 113 (13), 94 (32); mass measurement, found 220.18258, calcd for $C_{15}H_{24}O$, 220.18260.

Table II. Chromatographic Data for Hernandulcin (1) and Related Compounds

	TLC R_f value[a]				t_r, min	
compd	I	II	III	IV	HPLC[b]	GC/MS[c]
1	0.40	0.64	0.39	0.35	7.21	26.54[d]
2	0.27	0.61	0.27	0.24	10.29	8.85[e]
3	0.60	0.61	0.60	0.50	5.06	7.08[f]
4	0.47	0.66	0.50	0.39	5.40	11.68[d]
5	0.26	0.57	0.26	0.39	8.43	26.98[d]
6	0.19	0.60	0.19	0.17	14.40	16.11[d]
7	0.42	0.70	0.45	0.37	6.28	28.59[d]
8	0.15	0.38	0.16	0.11	8.12	26.37[d]

[a] Solvent systems: I, petroleum ether (bp range 60–80 °C)–acetone (9:1); II, chloroform–methanol (49:1); III, toluene–acetone (9:1); IV, cyclohexane–acetone (9:1). [b] Conditions: Hi-Chrom reversible Pirkle 1-A column, 25 cm × 46 mm (i.d.), particle size, 5 μm (Regis Chemical Co., Morton Grove, IL); solvent for elution, hexane–isopropyl alcohol, 19:1, at flow rate 1 mL/min; pressure, 660 psi; samples (2-pg portions) dissolved in 10 μL of eluting solvent, with 1 μg loaded onto column; detection at 220 nm. [c] Analysis: injection of 1 μL of 10% w/v solution of each compound in hexane onto a DB-5 fused silica capillary column (J&W, Rancho Cordova, CA), 30 m × 0.25 mm (i.d.); film thickness, 0.25 μm; carrier gas, helium at a linear velocity of 30 cm/s, with column head pressure of 10 psi and flow rate of 2 mL/min. GC/MS interface maintained at 180 °C, with the injector temperature at 80 °C, the ion source temperature operating at 120 °C with the column maintained isothermally for 3 min at 50 °C and then programmed at 6 °C/min to 300 °C. [d] Mass spectrum similar to electron-impact direct-probe data listed in text. [e] Significant ions observed in mass spectrum: m/z (relative intensity) 110 (M^+, 44%), 82 (100), 57 (11), 54 (35). [f] Significant ions observed in mass spectrum: m/z (relative intensity) 126 (M^+, 20%), 111 (33), 108 (91), 69 (84), 58 (25), 55 (100).

Thermal Dissociation of 1. A sample of naturally occurring (+)-hernandulcin (100 mg) was distilled at 140 °C under vacuum (0.15 mmHg), using a short-path distillation head. GC/MS analysis (see below) of the resultant clear distillate (89 mg) revealed that it was a mixture of two components. These compounds were separated by preparative TLC and identified as 3-methyl-2-cyclohexen-1-one (2) and 6-methyl-5-hepten-2-one (3) by comparison of their chromatographic behavior (TLC, GC) and spectral characteristics (UV, IR, NMR, MS) with those of authentic samples (Aldrich Chemical Co., Milwaukee, WI).

Chromatographic Parameters of Compounds 1–8. A summary of analytical chromatographic data (TLC using four solvent systems, HPLC, GC/MS) is presented, with relevant conditions, in Table II.

Acute Toxicity Studies. Experiments were carried out with male Swiss-Webster mice, housed in temperature-controlled rooms with a 12-h light/dark cycle, that were allowed free access to water and food. After acclimatization for 3 days, groups of 10 animals received a single dose of the test compound or plant extract by oral intubation. Test materials were dispersed in 1% aqueous sodium (carboxymethyl)cellulose, with a control group treated with 1% sodium (carboxymethyl)cellulose being included in each experiment. The *L. dulcis* combined leaf and flower petroleum ether soluble residue and compounds 1 (synthetic), 3, and 5 were tested at 1.0 and 2.0 g/kg of body weight, while compound 2 was evaluated at 1.1, 1.3, 1.4, 1.6, 2.0, and 2.4 g/kg of body weight. Mortality was determined up to 24 h after administration, and LD_{50} values were calculated graphically by the method of Miller and Tainter (1944). For test materials for which no mortality was recorded, animals were observed for 14 days to detect any delayed toxicity. Body weights were recorded on days 0 (prior to administration), 1, 3, 7, and 14. Body weight variations between treated and control groups were analyzed by use of one-way analysis of variance.

Bacterial Mutagenesis Assays. Experiments were performed with *Salmonella typhimurium* strain TM677, carrying the "R-factor" plasmid pKM101. The forward mutation assays were carried out by the procedure described by Pezzuto et al. (1985). These experiments were conducted in the presence and in the absence of a 9000*g* supernatant fraction (S-9) derived from the livers of Aroclor 1254 pretreated rats. The test materials were dissolved in dimethyl sulfoxide (20 μL) to give final concentrations of 0.31, 0.65, 1.25, 2.5, and 5.0 mg/mL. Materials tested for mutagenicity comprised the *L. dulcis* leaf–flower petroleum ether extract and compounds 1 (synthetic), 2, 3, and 5.

Molecular Mechanics Calculations. The structures of compounds 1, 5, and 8 were constructed by use of cyclohexane and other hydrocarbons contained in the library of the modeling software package CHEMLAB (Hopfinger and Pearlstein, in press). This input provided approximate structures with standard valence geometry, which were later optimized by MMFF molecular mechanics calculations. After calculation of partial atomic charges for this structure, using the CNDO/2 method, a fixed valence geometry conformational search at 30-deg increments about the C(2′)–C(3′), C(3′)–C(4′), and C(4′)–C(5′) bonds was made by use of steric, electrostatic, and hydrogen bond energy terms. Interatomic distances and torsional angles were calculated for the lowest energy conformer identified in this analysis. The MMFF program (Mabilia et al., 1985) used for this conformational analysis is a version of Allinger's MM2 program (Allinger, 1977), with extended parameterization.

RESULTS AND DISCUSSION

(+)-Hernandulcin (1) was isolated from a petroleum ether soluble extract of the leaves and flowers of *L. dulcis* in a total yield of 0.004% w/w of the dried plant material. Purification of 1 involved taste-guided fractionation, although this was carried out on a *L. dulcis* extract that proved to be both nonmutagenic and nontoxic for mice, when tested according to the procedures used in this study. The nonpolar nature of the sweet principle of *L. dulcis* demonstrated here thereby confirms the observations of earlier studies on this plant (Brodie and Jungk, 1881; Rio de la Loza, 1982; Breitwieser, 1943).

The structure of 1 was elucidated by a consideration of its spectroscopic properties, its degradation profile, and a synthetic method described here involving a directed aldol condensation. In its IR spectrum, 1 exhibited typical absorptions for a hydrogen-bonded hydroxyl group and an α,β-unsaturated carbonyl system. The latter functionality was confirmed by the observed maximum at 236 nm in the UV spectrum. Analysis of the ^1H NMR spectrum of 1 (Compadre et al, 1985) showed the presence of four methyl groups in the molecule, three of which were vinylic in nature. The spectrum exhibited signals for two olefinic protons, with one of them (s at δ 5.88) being assigned to the α-position of a conjugated carbonyl group. By analogy to a previous report in the literature (Bohlmann et al., 1976), this proton and a methyl resonance at δ 1.97 were considered components of a $C(CH_3)=CHC=O$ moiety. The other olefinic proton appeared as a triplet at δ 5.19 and, along with two of the vinyl methyl groups (δ 1.68 and 1.63), was postulated as being part of a $(CH_3)_2C=CHCH_2$ grouping. These findings, and high-resolution mass spectral measurements that indicated a molecular formula consistent with the elemental formula $C_{15}H_{24}O_2$, allowed the tentative identification of 1 as a sesquiterpene of the bisabolane class with the structure, 6-(1,5-dimethyl-1-hydroxyhex-4-enyl)-3-methylcyclohex-2-enone. The isom-

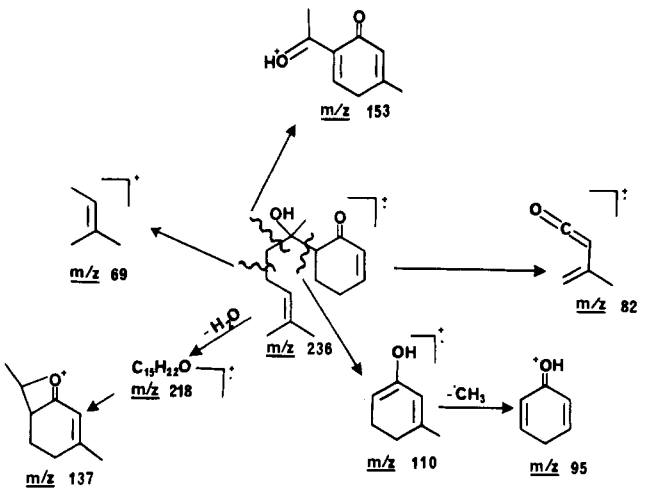

Figure 1. Mass spectral fragmentation pattern of hernandulcin (1).

eric structure, 5-(1,5-dimethyl-1-hydroxyhex-4-enyl)-2-methylcyclohex-2-enone, that corresponds to the known sesquiterpene delobanone (Takeda et al., 1971) was ruled out on the basis of the strong intramolecular hydrogen bonding observed in 1 and because the chemical shifts of the proton affixed to the conjugated double bond varied from 5.88 ppm in 1 to 6.20 ppm in delobanone.

Analysis of the high-resolution mass spectrum of 1 suggested a fragmentation pattern consistent with the structure proposed. The major pathways of fragmentation of 1, depicted in Figure 1, are the retro Diels–Alder rupture of the cyclohexenone ring to produce the fragment ion at m/z 82 and the fragmentation by retroaldolization of the C(6)–C(1′) bond to produce the base peak of the mass spectrum at m/z 110. Analogous processes have been reported for related bisabolane sesquiterpenes (Bohlmann et al., 1974; Yosioka et al., 1976). The fact that at elevated temperatures 1 suffered a reversed aldol condensation, producing a mixture of 2 and 3, provided further confirmatory evidence of the structure proposed for 1.

The directed aldol condensation of 2 and 3, using lithium diisopropylamide, occurred with about 50% completion, producing a mixture of the two diastereomeric products 1 and 5 in an approximate ratio of 19:1, plus small amounts of the dimeric products 4 and 6. The highly stereoselective outcome of the reaction suggested that (±)-hernandulcin is a mixture of the *R,R* and the *S,S* enantiomers and (±)-epihernandulcin is a mixture of the *R,S* and *S,R* counterparts. The rationale for this stems from the fact that ketone 2 would react via a chairlike intermediate having its bulkier substituent in an equatorial position (Evans et al., 1982; Mukaiyama, 1982). This, therefore, leads to the threo (*R,R*; *S,S*) isomers.

The dimeric compounds 4 and 6 were isolated as minor byproducts of self-condensation of the starting materials 3 and 2, respectively, and were characterized by their spectral characteristics.

The chemical shifts in the ^{13}C NMR spectra of 1 and 5 (Table I) were very similar, except for carbons C-5, -6, -2′, and -8′. Such discrepancies support the relative configurations assigned to 1 and 5 and were correlated with variations in the degree of intramolecular steric interaction in the molecular mechanics calculated preferred conformation of these compounds (Figure 2). Steric interactions mostly arise by the overlapping of van der Waals radii of closely spaced hydrogens. The steric perturbation of the C–H bond involved causes the charge to drift toward the carbon and usually causes a shielding effect of the carbons

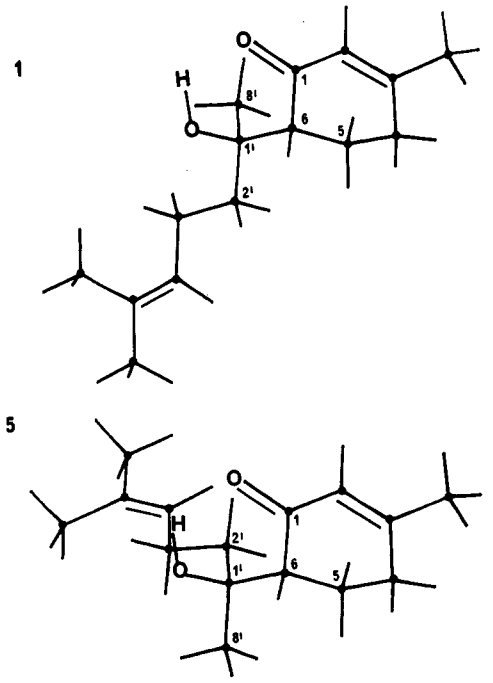

Figure 2. Preferred conformations of hernandulcin (1) and epihernandulcin (5) as predicted by molecular mechanics calculations.

attached to these hydrogens (Breitmaier and Voelter, 1978). These effects have been correlated with conformational variations of related β-ketol systems (Heathcock et al., 1979). When the ^{13}C NMR spectrum of 1 was compared with that of 5, a 3.4 ppm shielding effect in 1 was evident in C-6 due to the gauche interaction of the bulkier C-2′ substituent on the hydrogen atom attached to C-6 of 1, while the 1.9 ppm shielding effect on C-8′ probably arises from a 1,3-diaxial interaction with C-5. In the case of 5, C-2′ and C-5 were shielded, respectively, 3.2 and 2.9 ppm relative to those signals in 1, most likely due to their 1,3-diaxial interaction (Figure 2).

We have previously pointed to additional evidence obtained by the observation of a 28% nuclear Overhauser effect (NOE) in the ^1H NMR spectrum of 5 that indicated that the C-8′ methyl protons and the C-6 proton are in a syn conformation (Compadre et al., 1985). No such NOE effects were evident for (+)-hernandulcin and indeed would not have been expected for the structure proposed.

Table II presents analytical TLC, HPLC, and GC/MS data for hernandulcin and its synthetic precursors and reaction products, as well as the acetylated derivative 7, and reduction product 8. It may be seen that compounds 1 and 5 were separable by all three methods. While hernandulcin (1) was not detectable by GC/MS with the conditions used to analyze the other terpenoid constituents of *L. dulcis* leaves and flowers (Compardre et al., 1986), modification of the experimental conditions in the present study to permit a shorter column residence time of 1 permitted its GC/MS detection with considerable peak shape and mass spectral distortion.

No mortalities were recorded when the synthetic compound 1 was administered by gastric intubation to mice at the doses tested (1.0 and 2.0 g/kg of body weight). Thus, when tested in a similar manner, the LD_{50} of natural (+)-hernandulcin would be expected to be in excess of 1 g/kg of body weight. According to the same protocols, compound 5 produced no mortalities at the doses tested (1 and 2 g/kg).

The mutagenic potentials of compounds 1 and 5 were evaluated in *S. typhimurium* strain TM677. The results

obtained indicated no mutagenic activity at the doses used.

As mentioned above, compound 1 is degraded when heated at a relatively high temperature, and the products of this process are 2 and 3. These compounds are also the synthetic precursors of 1. Additionally, it is possible that these products could be generated from hernandulcin at lower temperatures upon long-term storage, especially under acidic or basic conditions (Mukaiyama, 1982), or could occur as impurites when being used as synthetic precursors of 1.

Compound 2 is a constituent of the oil of *Mentha pulegium* (Furia and Bellanca, 1975) and has also been isolated from the Douglas fir beetle, *Dendroctonus pseudotsugae* (Vité et al., 1972). This compound is used as a flavoring agent in a variety of products including candies, dairy products, baked goods, and nonalcoholic beverages and has a "generally regarded as safe" (GRAS) regulatory status (Arctander, 1969; Furia and Bellanca, 1975). Compound 3 also has a GRAS regulatory status and is a natural aroma component of many common edible items such as cocoa, coffee, and the lemon and lemongrass oils; it is used in the composition of numerous flavors and fragrances (Arctander, 1969; Furia and Bellanca, 1975). Since no reports describing their toxicity or health effects were found in the literature, the acute toxicity and mutagenicity of these compounds were studied in the present investigation.

The results of the evaluation of the acute toxicity for mice of compound 3 indicated no mortality at the doses studied (1 and 2 g/kg of body weight), suggesting an LD_{50} greater than 2 g/kg of body weight. Analysis of the treated animals' body weight variations for 14 days following compound administration demonstrated no significant difference upon comparison with a control group. However, some deaths were recorded in the case of 2, and its LD_{50} was calculated to be 1.6 ± 0.13 g/kg. Interestingly, the animals dying from the treatment with 2 presented similar toxic signs to the ones reported for the related 2-cyclohexen-1-one, e.g., convulsions and arching of the back (Levin et al., 1972). However, the toxicity determined for 2 is considerably lower than the reported toxicity of 2-cyclohexen-1-one (0.3 g/kg), and this is perhaps an indication of the involvement of the α,β-unsaturated carbonyl moeity, which, in the case of 2, is sterically hindered.

When evaluated for mutagenic potential with *S. typhimurium* strain TM677, compounds 2 and 3 exhibited no significant activity at the doses studied (0.31–5.0 mg/mL).

The two nonsweet hernandulcin derivatives, 7 and 8, have been fully characterized spectroscopically in the present study. Compound 7 was produced by treatment of 1 with acetic anhydride in the presence of 4-(dimethylamino)pyridine and exhibited the expected spectral data relative to those of 1. Compound 8 was obtained as the major product of the hydride reduction of 1, and its spectroscopic data suggested that the C-1 and C-6 hydrogen atoms are arranged in a cis manner, an assignment in agreement with expected data for the molecular mechanics calculated preferred conformation of the cis isomer, in which the torsional or dihedral angle between H-1 and H-6 is 49°. By contrast, in the calculated preferred conformation of the trans isomer, this angle is close to 180°. The strong upfield shift observed for C-5 in the ^{13}C NMR spectrum of 8 relative to that of 1 (Table I) is consistent with that which might be produced by a 1,3-diaxial interaction of the axially oriented hydroxyl group of a cis isomer, and it would be more unlikely in the case of a trans isomer, where the hydroxyl group is arranged in an equatorial position.

Molecular mechanics calculations determined in this investigation have revealed that the C-1′ hydroxyl and the C-1 carbonyl groups are arranged about 2.6 Å apart in the preferred conformation represented by hernandulcin (1; Figure 2). Therefore, this compound closely fits the model of Shallenberger et al. (1969) for sweet-tasting compounds.

We have already pointed out that, because of the low natural abundance of (+)-hernandulcin in *L. dulcis* (0.004% w/w), the future evaluation of hernandulcin as a sweetener will be more easily performed on the synthetic racemic form rather than on the naturally occurring compound (Compadre et al., 1986). While the sensory and hedonic parameters of (±)-hernandulcin (1) have not yet been established, the sweetness potency of this racemic substance is appreciably less than its (+) enantiomer, as would be reasonably expected because of the strict stereochemical requirements of the sweet receptor. The recent synthesis of (+)-hernandulcin (1; Mori and Kato, 1986) also offers the opportunity to further evaluate this substance, which has so far been found to be nonmutagenic, not acutely toxic for mice, and intensely sweet. The discovery of this prototype member of a new class of highly sweet compounds underscores the value of continuing to search the plant kingdom for further examples of intensely sweet molecules.

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Some Functional and Nutritional Properties of Oat Flours as Affected by Proteolysis

Rathy Ponnampalam,* Gilles Goulet, Jean Amiot, and Germain J. Brisson

Oat flour was hydrolyzed with Alcalase (A) and Neutrase (N) to the following degree of hydrolysis (DH): 3.8 (A1), 7.6 (A2), 6.0 (N1), 10.4% (N2). The functional and nutritional properties of the resulting products were compared to those of control oat flours and commercial whole wheat and soy flours. Water hydration capacity and isoelectric solubility increased with increase in DH. Proteolysis reduced emulsifying stability, fat-binding capacity, and heat coagulability when compared to controls. These properties were comparable or higher when compared to soy flour, except for A2. Water-soluble proteins had fragments of 925, 645, 395, and 230 molecular weights in Alcalase-treated and 835, 565, 340, and 195 molecular weights in Neutrase-treated oat flours. No significant differences were observed in the protein efficiency ratio (PER) among controls and enzyme-modified oat flours. There was a significant ($p < 0.05$) but small decrease in the nitrogen apparent digestibility coefficient in Alcalase-treated oat flours.

Oat is an inexpensive source of good quality protein with high protein content (Youngs et al., 1982). Although oat is nutritionally superior to other cereals, only 2.3% of the total oat crop harvested is used for human consumption in Canada (Statistics Canada, 1985), mainly as rolled oat groats (breakfast cereals) or oat flour (bakery products and infant foods). High-protein oats have been proposed for the enrichment of conventional foods (D'Appolonia and Youngs, 1978) and for the development of unconventional products such as high-protein beverages (Cluskey et al., 1976). In spite of the good nutritional qualities of oats, lack of information on the functional properties appears to be the main constraint to increased oat utilization.

The functionality of processed domestic and wild oat groat products was determined by Chang and Sosulski (1985). The nitrogen solubilities of domestic and wild groat products were very low in the pH region around the isoelectric point (Chang and Sosulski, 1985), but this was due to the steam treatments used to inactivate lipase. Although oat protein concentrates and isolates have relatively high fat-binding capacities, the nitrogen solubility is poor at neutral and slightly acidic pH (Ma, 1983a,b). One approach to improve the nitrogen solubility and expand the range of functional properties offered by oat flours, concentrates, and isolates is enzyme modification.

Enzyme modification has been used extensively to improve the functional properties of proteins and to tailor the functionality of certain proteins to meet specific needs (Richardson, 1977). Proteolytic enzymes have been employed to solubilize plant proteins from various sources such as soy (Puski, 1975; Mohri and Matsushita, 1984), peanut (Beuchat et al., 1975), rapeseed (Hermansson et al., 1974), and oat (Ma, 1985).

Some of the most important protein functional properties are solubility, emulsification, gelation, water hydration and fat-binding capacities, viscosity, heat coagulability, and foaming. Proteolysis alters these properties by changing the molecular size, conformation, solubility, and strength of the inter- and intramolecular bonds of the protein molecules (Kinsella, 1976; Ryan, 1977).

Uncontrolled or prolonged proteolysis, however, most often results in the formation of bitter peptides and products with undesirable functional properties. The proteolysis reaction must, therefore, be carefully monitored and controlled in order to manufacture ingredients with the desired "degree of hydrolysis" (DH), as this parameter is the key to product quality (Adler-Nissen, 1982). The parameter DH is defined as the percentage of peptide bonds cleaved (Adler-Nissen, 1976). When the hydrolysis is carried out at pH 7 or above, DH can be conveniently monitored by the pH-stat technique: the amount of base, which is added to the reaction mixture to keep the pH

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