Bitter Sesquiterpene Lactones from Chicory Roots

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Five known and one new sesquiterpene lactones were isolated from fresh chicory roots (*Cichorium intybus*). The new compound 11(*S*),13-dihydrolactucopicrin was identified by UV, ^{1}H NMR, ^{13}C NMR, and mass spectroscopy. The extraction procedure and HPLC analysis of sesquiterpene lactones in chicory were improved. The bitterness detection thresholds of all compounds were individually determined by triangle testing. 11(*S*),13-Dihydrolactucopicrin was extremely bitter, with a threshold level of 0.2 ppm. Four other lactones showed threshold values similar to that of quinine hydrochloride.

Chicory (*Cichorium intybus*) roots are inevitably produced by the cultivation and production of the edible chicory leaves. At present they are a waste. As the roots contain both the valuable fructose polymer inulin and some bitter sesquiterpene lactones, a research project of the Department of Food Science of Wageningen Agricultural University in the past years has been to explore possible uses of the otherwise useless roots. One possibility that is being investigated is the use of enzymatically liquefied and saccharified roots as a bittersweet starting material in the manufacture of soft drinks similar to tonic.

Lactucin and lactucopicrin have long been known to be at least partly responsible for the bitterness in chicory. Schenck and Graf (1939) determined the lowest concentration at which these compounds are perceived as bitter: ca. 2.0 and 1.7 ppm, respectively. Later Rees and Harborne (1985) claimed that 8-deoxylactucin is also a bitter compound, without mentioning any details. Kuusi and Autio (1985) and Voirol et al. (1987) have investigated only the total bitterness of a chicory root extract obtained by extraction with water.

One of us has earlier reported on HPLC analysis of chicory sesquiterpene lactones (Leclercq, 1984) and the release from chicory roots of sesquiterpene lactones (Leclercq and Netjes, 1985b) and inulin (Leclercq and Hageman, 1985a). In these earlier HPLC studies some major peaks in the chicory chromatogram remained unidentified (Leclercq, 1984).

In continuation of the earlier studies we therefore tried to isolate all the major sesquiterpenes present, to identify them by modern spectroscopic methods, to assign chemical structures to all the peaks in the chromatogram, and to improve on the existing rather elaborate extraction procedures and the HPLC separation. One more aim was the determination of the bitterness threshold value for each sesquiterpene lactone individually. In the following we report on these investigations.

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EXPERIMENTAL SECTION

Chicory roots were obtained from a grower in Veenendaal, The Netherlands, and stored 1 week at 1 °C before use.

Water Content. The water content was determined by weighing after drying 24 h at 70 °C in a hot-air oven with forced ventilation and by freeze-drying a portion of fresh roots. Both methods gave a water content of 86%.

Extraction Procedure. As far as was practically possible the extraction and separation were carried out with exclusion of natural or artificial light, i.e., by lowering of the sunscreens, avoiding direct artificial light, and covering of flasks etc. in aluminum foil.

Solvents. Solvents used for HPLC analysis were of HPLC grade or analytical grade. Solvents used for the extraction were of "synthetic" grade and were distilled before use.

Fresh chicory roots (8 kg) were cleaned and cut in small pieces, passed through a sieve (3.5-mm pore size), and liquefied at pH 4.25–4.75 at 37–42 °C with 8 g of Rapidase C600 (Gist Brocades, Delft, The Netherlands) for 2 h. After centrifugation (5000 rpm, 15 min) the 5-L supernatant solution was twice extracted in a separatory funnel with 1.3 and 1.1 L of ethyl acetate. The two ethyl acetate layers were combined, dried, and evaporated in vacuo. The residue (5.5 g) was extracted first with a mixture of 60 mL of ethyl acetate and 60 mL of methanol and next with a mixture of 20 mL of chloroform and 20 mL of ethanol. The two extraction liquids were combined and evaporated in vacuo. The new residue was treated with 50 mL of acetone, and the acetone-soluble fraction was filtered over a sintered glass disk and evaporated in vacuo (3.6 g). The two remaining insoluble residues, which were water soluble, were not further investigated. The acetone-soluble acetone fraction containing the crude sesquiterpene lactones was investigated by means of TLC and HPLC.

TLC. The following six solvent systems were tested in combination with ready-made silica gel TLC plates (60 F-254, Merck, no. 5719): (1) ethyl acetate = 100; (2) butyl acetate–ethyl acetate–1-butanol–acetic acid = 70:10:8:12; (3) butyl acetate–1-butanol–acetic acid–water = 85:15:40:20; (4) acetone–dichloromethane = 1:2; (5) hexane–chloroform–methanol = 45:45:10; (6) *tert*-butyl methyl ether = 100.

The following three solvent systems were tested in combination with ready-made reversed-phase TLC plates (Merck RP-18 F-254S, no. 15685): (1) methanol–water = 6:4; (2) acetonitrile–water = 5:5; (3) tetrahydrofuran–water = 45:55.

For both types of plates detection was carried out by viewing under 254-nm ultraviolet light.

0021-8561/90/1438-1035$02.50/0

Analytical HPLC. The best solvent for the separation of the sesquiterpene lactones was determined according to the optimization method of Glajch (1980). Both normal-phase HPLC on silica gel and reversed-phase HPLC (RP-HPLC) were tried.

Normal-phase HPLC was carried out on a 250 × 4.6 mm column filled with Lichrosorb Si (no. 28895), particle size 10 μm. The following three solvents were used: (1) dichloromethane–methanol = 95.5:4.5; (2) ethyl acetate–hexane = 75:25; (3) *tert*-butyl methyl ether–methanol = 99:1.

RP-HPLC was carried out on a 200 × 4.6 mm column filled with Spherisorb C18, particle size 5 μm. The following three solvents were used: (1) methanol–water = 1:1; (2) acetonitrile–water = 35:65; (3) tetrahydrofuran–water = 35:65. The flow was 1 mL/min in all cases. UV detection at 258 nm was used.

Separation Procedure. The crude sesquiterpene lactone fraction was separated in three portions (1.2 g each) on a Jobin Yvon 50 × 4 cm Modulprep column filled with 250 g of Merck Kieselgel 60 for preparative TLC as the stationary phase. As solvent pure distilled ethyl acetate at a flow of 18 mL/min was used. RI detection was used for recording the separation. Fractions of ca. 15 mL were collected with a Gilson 202 fraction collector. UV-active fractions were further checked with TLC and HPLC (see below). Three fractions, which according to RP-HPLC consisted each of two compounds, were obtained.

Fraction 1 was further separated into 11(*S*),13-dihydrolactucopicrin and lactucopicrin by preparative HPLC on a Rainin 250 × 10 mm column (no. C18-80-299-C5) filled with Microsorb C18, particle size 5 μm, with 1:1 methanol–water as solvent at 4 mL/min. Fraction 3 was separated into 11(*S*),13-dihydrolactucin and lactucin on the same column with 3:7 methanol–water as solvent at 4 mL/min. Fraction 2 was separated into 11(*S*),13-dihydro-8-deoxylactucin and 8-deoxylactucin by semi-preparative HPLC on the analytical column with 1:1 methanol–water as solvent at 1 mL/min. The ca. 400 necessary injections were carried out automatically by a Gilson isocratic Autoprep system controlled by an Apple computer.

Spectroscopic Apparatus. ^{1}H NMR spectra were recorded in $CDCl_3$ or $CDCl_3$–CD_3OD on a Bruker CXP300. The $CHCl_3$ signal was used as an internal reference (7.26 ppm). Assignments were confirmed by homonuclear decoupling experiments or two-dimensional NMR. ^{13}C NMR spectra were recorded in $CDCl_3$ or $CDCl_3$–CD_3OD on a Bruker CXP300. The $CDCl_3$ signal was used as an internal reference (77 ppm). Mass spectra were obtained on an AEI MS-902 at 70 eV in the electron impact mode.

Spectral data for 11(*S*),13-dihydrolactucopicrin are as follows. UV (MeOH) λ_{max}: 255, 230 nm (sh). MS (70 eV) *m/z* (relative intensity): 412 (M^+, 1), 322 (1), 260 (8), 231 (6), 198 (19), 187 (48), 152 (41), 107 (100). ^{1}H NMR (300 MHz, $CDCl_3$) δ 1.20 (H-13, d, *J* = 7 Hz), 2.32 (H-7, ddd, *J* = 11.5, 10.4, 10.3 Hz), 2.36 (H-9β, dd, *J* = 13.7, 2.5 Hz), 2.44 (H-15, s), 2.46 (H-11, dq, *J* = 11.5, 7 Hz), 2.70 (H-9α, dd, *J* = 13.7, 10.7 Hz), 3.55 (H-α′, d, *J* = 15 Hz), 3.58 (H-5, br d, *J* = 10 Hz), 3.61 (H-α″, d, *J* = 15 Hz), 3.69 (H-6, dd, *J* = 10.3, 10 Hz), 4.53 (H-14a, br d, *J* = 17.6 Hz), 4.82 (H-8, ddd, *J* = 10.8, 10.4, 2.5 Hz), 4.85 (H-14b, br d, *J* = 17.6 Hz), 6.44 (H-3, m), 6.81 (H-3′, H-5′, d, *J* = 9 Hz), 7.13 (H-2′, H-6′, d, *J* = 9 Hz). ^{13}C NMR (75 MHz, $CDCl_3$–CD_3OD) δ 14.5 (C-13), 21.2 (C-15), 40.5* (C-11), 40.8* (C-α), 44.5 (C-9), 48.8 (C-5), 58.5 (C-9), 61.9 (C-14), 70.6 (C-8), 80.6 (C-6), 115.5 (C-3′), 115.5 (C-5′), 123.8 (C-1′), 130.1 (C-2′), 130.1 (C-6′), 132.5§ (C-1), 133.1§ (C-3), 146.5 (C-10), 156.1 (C-4′), 171.0 (C-β), 172.8 (C-4), 177.0 (C-12), 195.0 (C-2).

Sensory Analysis. (*1*) *Threshold Determinations.* A professional panel of 16 housewives (40–55 years) employed by Quest International was used to determine threshold values. Samples were presented as 50-mL aqueous solutions in plastic serving cups. The concentrations used for each of the lactones are indicated in Table I. The panel was asked to select the one cup, out of three cups, that contained a stimulus other than water. The order of presentation for each of these triangle tests was balanced over all panelists: AAB, ABA, BAA. Evaluations were conducted at room temperature in a darkened room to minimize the chance of instability; see, e.g., Leclercq et al. (1988).

The data were analyzed by using a two-segment model for forced ternary choice, single trial. This model calculates an estimate, α, of the proportion of panelists who could discriminate between the stimulus and a blank, based on the proportion of correct answers. The threshold value (α = 0.5) was obtained by linear regression of α on concentration.

Table I. Concentrations Used in the Sensory Experiments and Thresholds Determined for the Six Sesquiterpene Lactones and Quinine Hydrochloride[a]

compd	concentration		threshold
lactucin	0.108	0.866	1.7
	0.217	1.732	
	0.433	3.464	
lactucopicrin	0.096	0.767	0.5
	0.192	1.534	
	0.384	3.069	
8-deoxylactucin	0.107	0.857	1.1
	0.214	1.713	
	0.428	3.426	
11(*S*),13-dihydrolactucin	0.094	0.751	1.4
	0.188	1.503	
	0.376	3.005	
11(*S*),13-dihydrolactucopicrin	0.096	0.764	0.2
	0.191	1.528	
	0.382	3.055	
11(*S*),13-dihydro-8-deoxylactucin	0.109	0.873	1.1
	0.218	1.747	
	0.437	3.493	
quinine hydrochloride	0.031	1.000	1.6
	0.063	2.000	
	0.125	4.000	
	0.250	8.000	
	0.500	16.000	

[a] Values are in ppm (w/w).

(*2*) *Intensity Functions.* Intensity functions were obtained for lactucin and dihydrolactucin, in addition to quinine hydrochloride, using six members of the above-mentioned panel. All panelists were PTC tasters, and they have been trained to use a fully automated audio method for intensity measurement (King, 1986). The data from each compound were averaged over the panel and fitted to power functions.

RESULTS AND DISCUSSION

In our earlier investigations a rather time-consuming extraction with diethyl ether was used (Leclercq, 1984), which would be impractical for the preparative isolation. The main problem is the extraction of lactucin, which shows good solubility neither in organic solvents nor in water. A number of solvents were investigated, and ethyl acetate was selected. A double extraction of an aqueous extract with ethyl acetate removed more or less all sesquiterpene lactones, including lactucin. This corresponds well with the data of Schenck et al. (1961), who also found that ethyl acetate was one of the best solvents for the extraction of lactucin. A large portion of chicory roots was treated with commercial pectolytic and cellulolytic enzymes and centrifuged after liquefaction. The liquefaction has the advantage that all the lactones are in solution and more easy to extract; i.e., they do not have to diffuse through cell walls. The filtrate was twice extracted with ethyl acetate, and the combined organic solvents were dried and evaporated.

The extract was investigated by both normal-phase and reversed-phase HPLC. Altogether ten solvents and three different columns were tested. The best results were given by a Spherisorb C18 5 μm column with the solvent 5:5 methanol–water. Six major peaks, all sesquiterpene lactones according to their UV spectrum, could be distinguished (Figure 1). The crude extract was first prefractionated by means of preparative LC on silica gel with ethyl acetate as solvent in three fractions, each consisting of two compounds. These were further purified by means of preparative HPLC on C18 columns with methanol–water mixtures into the six sesquiterpene lactones.

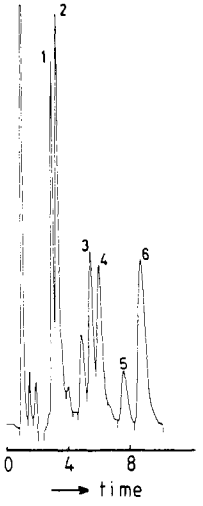

Figure 1. HPLC trace of a crude ethyl acetate extract from chicory roots: 1 = 11(*S*),13-dihydrolactucin, 2 = lactucin, 3 = 11(*S*),13-dihydro-8-deoxylactucin, 4 = 8-deoxylactucin, 5 = 11(*S*),13-dihydrolactucopicrin, 6 = lactucopicrin.

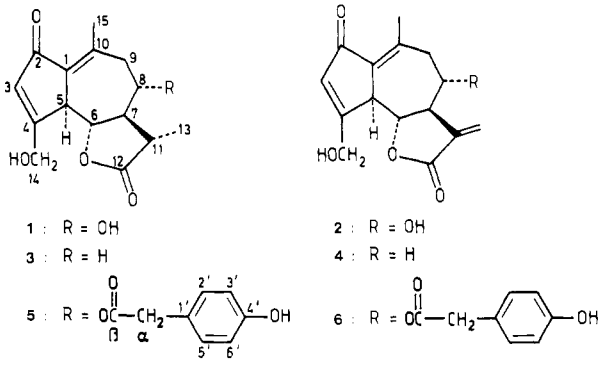

Figure 2. Structures of the six sesquiterpene lactones identified in chicory roots.

The pure compounds were identified by means of UV, mass, ^{1}H NMR, and ^{13}C NMR spectroscopy. Compounds **2**, **4**, and **6** (Figure 1) were identified as lactucin (**2**), 8-deoxylactucin (**4**), and lactucopicrin (**6**). Structures are given in Figure 2. These compounds have been identified before in chicory (Pyrek, 1985).

Compound **1** was identified as 11(*S*),13-dihydrolactucin, a known compound (Sarg et al., 1982; Mahmoud et al., 1986; Nishimura et al., 1986; Seto et al., 1988) but not previously isolated from *Cichorium intybus*. The stereochemistry at C-11 was unambiguously determined as *S* by means of a comparison of the ^{1}H NMR and ^{13}C NMR data with those of similar compounds and NOE difference experiments. The observed coupling between H-7 and H-11 of 12.0 Hz in the ^{1}H NMR spectrum is typical of the *S* stereochemistry (ca. 7 Hz for the 11*R* isomer). In the NOE difference experiments irradiation of the H-11 proton gave NOE's for H-6, H-8, and H-13. Irradiation of the H-13 protons gave NOE's for H-7, H-11, and H-9α. These results can be only explained by an α-methyl group (11*S*).

Compound **3** was identified as 11(*S*),13-dihydro-8-deoxylactucin (syn. jacquinelin) and has been detected before in *Cichorium intybus* (Pyrek, 1977). The stereochemistry was determined as 11*S* by the chemical shift of 2.26 ppm for H-11 (ca. 2.70 ppm for 11*R*) and the coupling constant of 12.5 Hz between H-7 and H-11 (ca. 7 Hz for 11*R*). In the ^{13}C NMR spectrum the shifts of C-7, C-8, C-11, and C-13 at 56.1, 25.9, 41.4, and 12.2 ppm, respectively, were in good agreement with those expected for such a compound (Adegawa et al., 1985; Martinez et al., 1988). Shifts of ca. 52, 24, 39, and 10 ppm, respectively, would have been expected for the 11*R* stereochemistry.

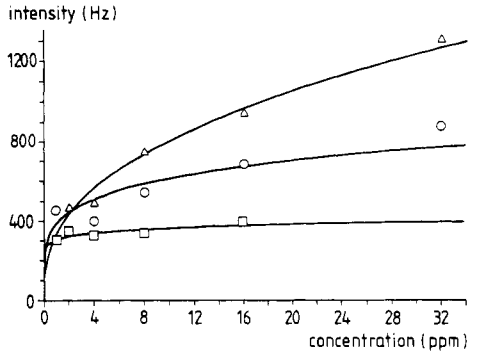

Figure 3. Intensity functions for bitterness: (△) dihydrolactucin; (○) quinine hydrochloride; (□) lactucin. The following power functions showing the relationship of intensity to concentrations were found: lactucin, intensity = 309[concn]$^{0.070}$, correlation = 0.778; 11(*S*),13-dihydrolactucin, intensity = 327[concn]$^{0.391}$, correlation = 0.982; quinine hydrochloride, intensity = 381[concn]$^{0.205}$, correlation = 0.850.

Compound **5** was identified as 11(*S*),13-dihydrolactucopicrin. Its molecular ion was observed at *m*/*z* 412, 2 mass units higher than M^+ of lactucopicrin. The fragments corresponding with the side chain, e.g., *m*/*z* 152, and the base peak at *m*/*z* 107 were observed at the same mass as in lactucopicrin while many other fragments were shifted 2 mass units. The ^{1}H NMR spectrum of **5** was in full agreement with the structure of 11(*S*),13-dihydrolactucopicrin, the H-13 protons of the methyl side chain were observed as a doublet (J = 7 Hz) at 1.20 ppm, H-8 was observed at the characteristic value of 4.78 ppm, and signals for all the protons of the *p*-hydroxyphenylacetic acid ester were also present. In the ^{13}C NMR spectrum C-13 was observed at 14.5 ppm, confirming the 11*S* stereochemistry.

Table I gives the threshold values obtained for the six lactones and quinine hydrochloride. The intensity functions for the two lactucin isomers and quinine hydrochloride are shown in Figure 3.

Lactucopicrin and dihydrolactucopicrin are more bitter than quinine hydrochloride; the other lactones have thresholds comparable to that of quinine hydrochloride. The bitter taste and occasional mouth coating could be sources of a carry-over effect, but a χ^2 test showed no significant correlation between placement of the odd sample in the triangle and correctness of its identification.

As can be seen from the exponent (see Figure 1), dihydrolactucin showed a greater increase in bitterness with increased concentration than either quinine hydrochloride or lactucin.

If lactucin is taken as a reference compound, it follows from the results that substitution at the β-position, especially by the 4-hydroxyphenylacetic acid group, lowers the bitterness threshold value. Reduction of the exocyclic methylene group of the α,β-unsaturated lactone ring also enhances the bitterness somewhat. It can be concluded that some variation in these two parts of the molecule has only a limited influence on the total bitterness of the molecule. Other parts of the molecule, e.g., the dienone system, may be more essential for the bitterness.

ACKNOWLEDGMENT

We thank J. de Mik for his gift of the chicory roots and Drs. C. Erkelens (Leiden University) for recording some NOE difference spectra of 11(*S*),13-dihydrolactu-

cin, A. van Veldhuizen for recording the NMR spectra, C. J. Teunis for recording the mass spectra, and S. Soekhai for help in carrying out the sensoric experiments.

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Received for review July 13, 1989. Accepted November 21, 1989.

Investigation of 2-Hydroxy-2-cyclopenten-1-ones in Roasted Coffee

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The weakly acidic volatile components of roasted coffee were analyzed on a GC/MS. Fourteen 2-hydroxy-2-cyclopenten-1-ones were identified. Ten of these compounds are being reported for the first time in roasted coffee. *trans*- and *cis*-2-hydroxy-3,4,5-trimethyl-2-cyclopenten-1-ones are new compounds. Possible precursors of the 2-hydroxy-2-cyclopenten-1-ones are discussed.

To date, 670 compounds have been identified in roasted coffee aroma (Flament and Chevallier, 1988). Four major 2-hydroxy-2-cyclopenten-1-ones were identified in roasted coffee (Gianturco et al., 1963). A mechanism for formation of 2-hydroxy-3-methyl-, 2-hydroxy-3,4-dimethyl-, 2-hydroxy-3,5-dimethyl-, and 3-ethyl-2-hydroxy-2-cyclopenten-1-one (**1**, **2**, **3**, and **8**, respectively) from hydroxy ketones is presented by Shaw et al. (1968). All four 2-hydroxy-2-cyclopenten-1-ones had strong caramel-like odors, **2** being the most powerful. In the present work, we report the identification and quantification of 14 2-hydroxy-2-cyclopenten-1-ones in roasted coffee.

EXPERIMENTAL SECTION

Materials. High-grown Arabica coffee beans of Colombian origin were roasted to a medium roast (205 °C, 12 min) and stored in packages with an excess of air at 4 or 25 °C. 2-Hydroxy-3-methyl-, 2-hydroxy-3,4-dimethyl-, 2-hydroxy-3,5-dimethyl-, and 3-ethyl-2-hydroxy-2-cyclopenten-1-ones (**1**-**3** and **8**) were commercially available (Seimi Chemical Co., Ltd., Hercules Inc., and Pfizer Inc.) All starting chemicals were obtained from reliable commercial sources and used without further purification.

Isolation of 2-Hydroxy-2-cyclopenten-1-ones. A 40-kg portion of roasted coffee was ground and subjected to steam distillation. The distillate (62.5 kg) was extracted with methylene

0021-8561/90/1438-1038$02.50/0