Characterization of Tricyclic Sesquiterpenes in Hop (*Humulus lupulus*, var. Hersbrucker Spät)

Roland Tressl,* Karl-Heinz Engel, Maria Kossa, and Hans Köppler

Investigations of hop variety Hersbrucker Spät by means of distillation–extraction, liquid–solid chromatography, and gas chromatography–mass spectrometry led to the characterization of a new class of hop constituents: tricyclic sesquiterpenes. More than 15 components (sesquiterpene hydrocarbons, epoxides, and alcohols) could be identified for the first time in hop, and the biogenetical relations of the new tricyclic sesquiterpenes to germacrenes, selinenes, and selinadienes are demonstrated. The new components are characteristic constituents of the variety Hersbrucker Spät, some of them were detected in the varieties Hüller Bitterer, Perle, and Hallertauer Mittelfrüh, but they are not contained in Saazer hop, Northern Brewer, or Brewer's Gold. So this study may be a further step to the differentiation of hop varieties.

The volatile constituents of hop were investigated by Buttery et al. (1965, 1966), who identified esters, ketones, and sesquiterpene hydrocarbones in American hop varieties. Naya and Kotake (1972) characterized sesquiterpenoids in Japanese hops. Tressl et al. (1978a,b) could confirm these results for German varieties and demonstrated that the oxygenated terpenes and sesquiterpenes of hop are transferred to some extent to beer. Some of our results were confirmed by Peacock and Deinzer (1981). The variety Hersbrucker Spät, which possesses many so far unidentified sesquiterpenes, is cultivated in the Hallertau in 1982 on an area of 4000 ha. The part of the variety Hallertauer Mittelfrüh which belongs to the so-called "aroma hops" is more and more decreasing because of deterioration by Poronospora. Both Hersbrucker Spät and Hallertauer Mittelfrüh contain low amounts of humulene and myrcene but possess different sesquiterpene spectra. Their investigation in the present study led to the characterization of a new class of hop constituents: tricyclic sesquiterpenes. Hersbrucker Spät contains more than 15 tricyclic sesquiterpenes which may be derived from bicyclogermacrene, selinadienes, selinenes, and germacrene B. These components are not contained in Saazer hop, Northern Brewer, or Brewer's Gold, but some of them were detected in the varieties Hüller Bitterer, Perle, and Hallertauer Mittelfrüh.

EXPERIMENTAL SECTION

Hops (*Humulus lupulus* var. Hersbrucker Spät) were harvested in the Hallertau and stored at 4 °C until analyzed.

Sample Preparation. One hundred grams of homogenized hops was mixed with 1200 mL of distilled water, and the hop oil constituents were isolated by means of distillation–extraction with pentane–ether (1:1) for 4 h (Krüger and Baron, 1975). The extract was dried over Na_2SO_4, concentrated to a volume of 2 mL, and separated by adsorption chromatography.

Adsorption Chromatography. Separation according to the polarity of the components was carried out by liquid–solid chromatography. One hundred microliters of hop oil was placed on a cooled column (200 × 9 mm i.d.) filled with 5 g of silica gel (activity II–III, Merck 7734). The hydrocarbons were eluted with 100 mL of (I) pentane and the oxygenated components with 100 mL of ether. Both fractions were concentrated to a definite volume by using a Vigreux column and analyzed by GLC. The oxygenated components were separated again into six fractions on silica gel and eluted with 40 mL of (I) pentane (P), (II) pentane–methylene chloride (P–MC) (9:1), (III) P–MC (2:1), (IV) P–MC (1:2), (V) P–ether (9:1), and (VI) ether. Each fraction was concentrated to a definite volume and examined by GLC and by capillary GLC–MS.

Gas Chromatography. *Preparative GLC.* Trace constituents were enriched by preparative GLC using a 3 m long × 4 mm i.d. glass column packed with 60-mesh Chromosorb WAW coated with 12% CW 20M. The column was programmed at 4 °C/min from 70 to 220 °C. At the outlet of the column the samples were split 1:10 (1 part to FID) and the eluted components collected in dry ice cooled Pyrex tubes (3 mm × 15 cm long).

Capillary GLC–Mass Spectrometry. Capillary GLC was carried out by using a 50-m glass capillary column (0.32-mm i.d.) coated with Carbowax 20M in a Carlo Erba Fractovap 2101 with linear temperature program from 70 to 180 °C at 2 °C/min (column A). For capillary GLC–mass spectrometry a 50-m glass capillary column (0.32-mm i.d.) coated with UCON (column B) in a Carlo Erba Fractovap 2101 was connected to a double-focusing mass spectrometer CH5-DF (Varian MAT, West Germany). Conditions were as follows: column temperature program from 70 to 180 °C at 2 °C/min, ionization voltage 70 eV, ion source temperature 200 °C, and resolution 2000 (10% valley).

Synthesis of Epoxides and Alcohols. Epoxides were synthesized by reaction of the corresponding hydrocarbon with an equimolar quantity of 3-chloroperbenzoic acid in dry ether at 0 °C over 24 h. The formed diastereomeric epoxides were reduced with $LiAlH_4$ in refluxing THF over 12 h. The complex LiAl–alcoholates were hydrolyzed with 10% sulfuric acid and the alcohols were obtained by extraction of the solution with ether.

Reference Compounds. Reference samples were purchased if available or were gifts from Professor Bohlmann (Berlin), Dragoco (Holzminden), and Haarman and Reimer (Holzminden).

RESULTS AND DISCUSSION

The hop oil was isolated by distillation–extraction, separated by liquid–solid chromatography, and investigated by capillary GC–MS as described (Tressl et al., 1978a,b). Trace constituents were further enriched by preparative GC. It can be seen from Figure 1 that the variety Hersbrucker Spät contains more sesquiterpene

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Table I. Sesquiterpenes of Hop (Variety Hersbrucker Spät)

hydrocarbons	LSC	I_K(CW-20M)	I_K(UCON)	concn, mg/kg of hop	identification
1, α-cubebene	I	1431	1409	5	MS[a]
2, α-ylangene	I	1452	1431	5	MS
3, α-copaene	I	1468	1435	15	MS[a]
4, α-gurjunene	I	1492	1467	11	MS[a,b]
5, β-cubebene	I	1533	1499	5	MS[b]
6, β-caryophyllene	I	1552	1514	520	MS[a]
7, aromadendrene	I	1560	1527	98	MS[a,b]
8, α-guaiene	I	1569	1533	16	MS[b]
9, γ-elemene	I	1582	1546	16	MS[b]
10, alloaromadendrene	I	1595	1555	77	MS[a,b]
11, β-farnesene	I	1627	1579	35	MS[a]
12, humulene	I	1620	1571	1200	MS[a]
13, γ-muurolene	I	1634	1591	170	MS
14, δ-selinene	I	1639	1593	55	MS[a,b]
15, viridiflorene	I	1645	1598	60	MS[b]
16, germacrene D	I	1645	1605	15	MS[a]
17, δ-guaiene	I	1664	1592	20	MS[a,b]
18, β-selinene	I	1665	1610	150	MS[a]
19, α-selinene	I	1670	1615	170	MS[a]
20, α-muurolene	I	1677	1621	35	MS
21, bicyclogermacrene	I	1675	1628	10	MS[a,b]
22, γ-cadinene	I	1704	1647	60	MS
23, δ-cadinene	I	1704	1647	60	MS
24, selina-4,7-diene	I	1720	1660	145	MS
25, selina-3,7-diene	I	1720	1665	195	MS
26, α-cadinene	I	1736	1676	10	MS
27, calamenene	I	1773	1748	2	MS
28, germacrene B	I	1766	1697	105	MS[b]
29, cadalene	I	1852		2	MS

[a] Comparison of retention and MS with those of the authentic sample. [b] Identified for the first time in hops.

hydrocarbones than the variety Northern Brewer (which is similar to Hallertauer Mittelfrüh). Myrcene, α-copaene, β-caryophyllene, humulene, α- and γ-muurolene, α- and β-selinene, and δ-cadinene are common constituents in all hop varieties. The concentrations of the α- and β-selinenes and of the selinadienes are strongly increased in Hersbrucker Spät. Selinadienes were not detected in Northern Brewer. Most of the constituents were identified by comparison of retention data and mass spectra of the isolated components with authentic samples. Some of the results are summarized in Table I.

α-Gurjunene, γ-elemene, δ-selinene, aromadendrene, alloaromadendrene, viridiflorene, α-guaiene, and bicyclogermacrene were characterized for the first time in hop. Their mass spectra are shown in Table II. Germacrene B was identified in hop by Hartley and Fawcett (1969). During its isolation by preparative GC germacrene B was transformed into γ-elemene and selina-3,7-diene as observed by Okamoto et al. (1981). Naya and Kotake (1972) identified germacrene D in ripening hops. This compound is known as a precursor of cadinene-type sesquiterpenes. The characterization of bicyclogermacrene and of germacrene B in this study seems to be of some relevance in the biogenesis of the tricyclic sesquiterpenes in Hersbrucker Spät. Bicyclogermacrene may be transformed by biogenetic-type rearrangements into α-gurjunene, aromadendrene, alloaromadendrene, and viridiflorene. Germacrene B is a precursor of γ-elemene and selinadienes which were characterized in the hop oil. The biosynthesis of sesquiterpene hydrocarbones is strictly correlated to the formation of alcohols. Therefore, we separated the oxygenated fraction of the hop oil by LSC into six fractions according to functional groups. Fraction III contained esters, fraction IV ketones (not investigated), and fractions V and VI sesquiterpenoids. Figure 2 presents the capillary GC separation of the LSC fractions V and VI which contained sesquiterpene epoxides and alcohols. The components were isolated by preparative GC and further investigated by capillary GC–MS. Some of the results are summarized in Table III. Caryophyllene epoxide, humulene epoxides I and II, caryolan-1-ol, epicubenol, T-

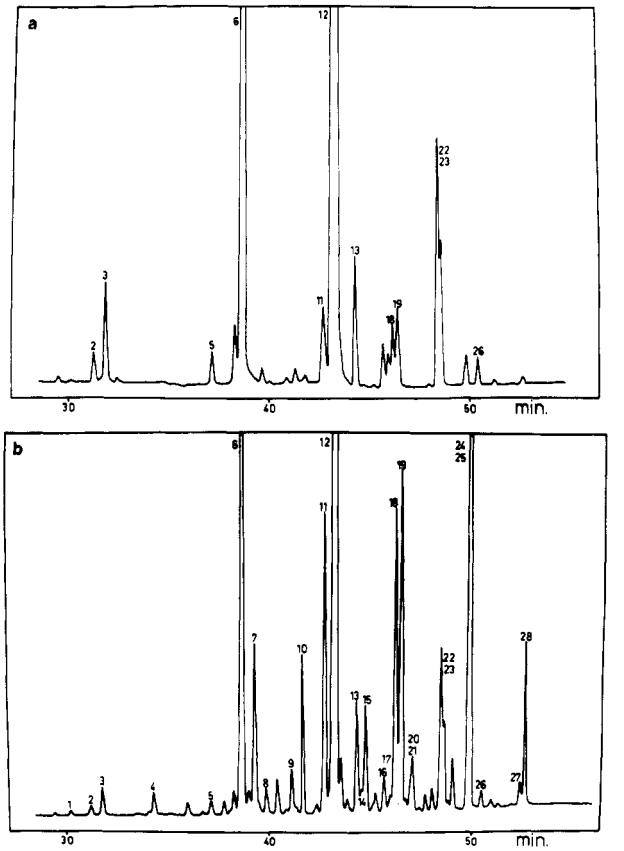

Figure 1. Gas chromatograms of LSC fractions I (sesquiterpene hydrocarbons) of hop varieties: (a) Northern Brewer; (b) Hersbrucker Spät. (Column A; peak numbers correspond to the component numbers in Table I.)

Table II. Mass Spectral Data of Those Components Identified for the First Time in Hop

component	M_r	mass spectral data, m/e (rel intensity)
hydrocarbons		
α-gurjunene	204	105 (100), 161 (76), 119 (71), 41 (67), 91 (64), 204 (53), 55 (48), 81 (47), 189 (47), 133 (45)
β-cubebene	204	161 (100), 105 (35), 91 (30), 41 (25), 120 (25), 119 (20), 81 (18), 79 (15), 55 (15), 93 (15)
α-guaiene	204	105 (100), 107 (72), 93 (68), 147 (60), 81 (52), 91 (51), 79 (51), 41 (47), 95 (41), 133 (40)
γ-elemene	204	93 (100), 121 (96), 41 (94), 105 (74), 107 (64), 55 (63), 91 (60), 67 (58), 79 (58), 81 (48)
aromadendrene	204	91 (100), 93 (93), 105 (85), 79 (80), 41 (73), 107 (73), 69 (71), 81 (71), 67 (68), 119 (67)
alloaromadendrene	204	91 (100), 41 (91), 105 (90), 79 (79), 93 (78), 107 (66), 67 (63), 81 (63), 69 (59), 55 (59)
viridiflorene	204	107 (100), 105 (96), 93 (90), 91 (66), 79 (57), 119 (55), 161 (53), 41 (53), 81 (50), 55 (41)
δ-guaiene	204	93 (100), 107 (97), 108 (86), 105 (72), 79 (66), 81 (57), 91 (57), 95 (48), 41 (49), 55 (41)
bicyclogermacrene	204	121 (100), 93 (81), 161 (51), 107 (53), 105 (47), 41 (43), 119 (39), 79 (39), 81 (36), 91 (31)
oxygenated components		
aromadendrene epoxide	220	91 (100), 81 (93), 93 (88), 79 (87), 67 (83), 41 (81), 95 (81), 55 (76), 105 (72), 107 (69)
alloaromadendrene epoxide	220	41 (100), 91 (93), 67 (85), 81 (80), 79 (75), 82 (70), 93 (70), 95 (66), 105 (64), 55 (64)
β-selinene epoxide I	220	107 (100), 81 (94), 95 (88), 93 (84), 41 (82), 67 (76), 79 (74), 55 (71), 43 (67), 109 (65)
epiglobulol	222	43 (100), 82 (80), 109 (67), 69 (64), 81 (50), 105 (50), 41 (50), 93 (49), 55 (42), 67 (42)
globulol	222	43 (100), 41 (56), 69 (56), 81 (56), 109 (47), 107 (45), 93 (44), 95 (41), 161 (38), 55 (38)
ledol	222	43 (100), 69 (85), 81 (78), 109 (68), 41 (66), 93 (63), 67 (62), 107 (62), 82 (55), 55 (53)
viridiflorol	222	43 (100), 69 (72), 109 (71), 41 (60), 81 (57), 105 (52), 93 (51), 161 (50), 107 (50), 67 (45)
cubenol	222	119 (100), 161 (57), 105 (55), 43 (42), 41 (38), 82 (37), 55 (35), 81 (34), 69 (27), 93 (25)
spathulenol	220	43 (100), 41 (61), 91 (55), 93 (47), 119 (46), 105 (39), 69 (38), 79 (37), 205 (35), 107 (32)
selin-11-en-4-ol	222	81 (100), 43 (90), 71 (73), 135 (55), 95 (54), 67 (50), 93 (50), 55 (46), 109 (43), 107 (41)
palustrol	222	43 (100), 41 (52), 55 (51), 111 (45), 67 (43), 69 (31), 93 (31), 83 (31), 81 (26), 95 (23)

Table III. Sesquiterpenes of Hop (Variety Hersbrucker Spät)

oxygenated components	LSC	I_K(UCON)	concn, mg/kg of hop	identification
1, aromadendrene epoxide	V	1807	20	MS[b,c]
2, caryophyllene epoxide	V	1807	24	MS
3, humulene epoxide I	V	1833	7	MS
4, alloaromadendrene epoxide	V	1840	12, 5	MS[b,c]
5, humulene epoxide II	V	1856	71	MS[a]
6, selinene epoxide I	V	1863	2	MS[b,c]
7, epiglobulol	VI	1826	5	MS[b,c]
8, ledol	VI	1847	8	MS[a,b]
9, caryolan-1-ol	VI	1854	5	MS
10, globulol	VI	1877	27	MS[a,b]
11, viridiflorol	VI	1886	16	MS[a,b]
12, epicubenol	V	1872	13	MS
13, cubenol	V	1881	17	MS[b,c]
14, spathulenol	VI	1912	10	MS
15, γ-eudesmol	VI	1918	18	MS[b]
16, T-cadinol	VI	1940	9, 5	MS
17, δ-cadinol	V + VI	1953	36, 5	MS
18, α-eudesmol	VI	1992	65	MS
19, β-eudesmol	VI	1997	60	MS[a]
20, α-cadinol	VI	2000	15	MS
21, selin-11-en-4-ol	VI	2007	29	MS[b,c]
22, humulenol II	VI	2024	15	MS
23, juniper camphor	VI	2043	13, 5	MS
24, palustrol	VI	2070	7	MS[b,d]
25, farnesol	VI	2122	17	MS[a]

[a] Comparison of retention and spectra with those of the authentic sample. [b] Identified for the first time in hop. [c] Synthesis. [d] Tentatively identified.

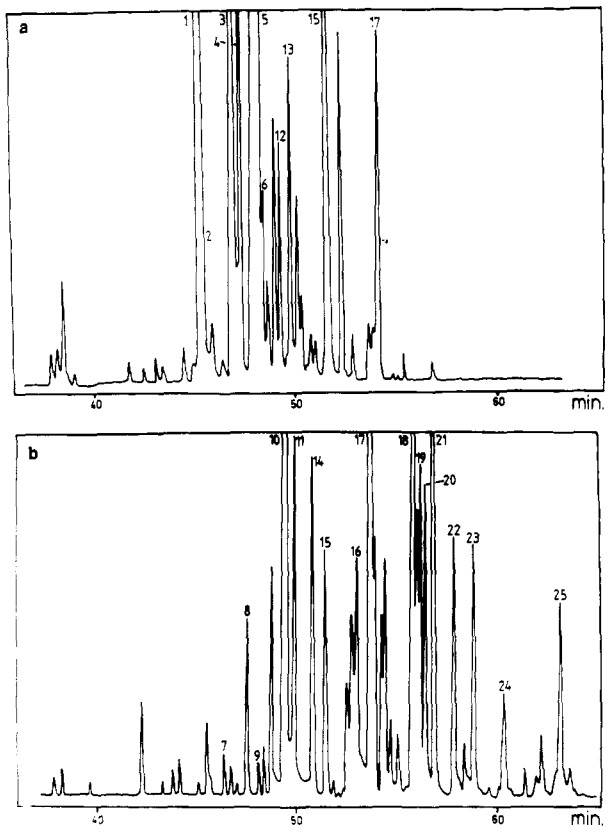

Figure 2. Gas chromatograms of the hop variety Hersbrucker Spät (sesquiterpene epoxides and alcohols): (a) LSC fraction V; (b) LSC fraction VI. (Column B; peak numbers correspond to the component numbers in Table II.)

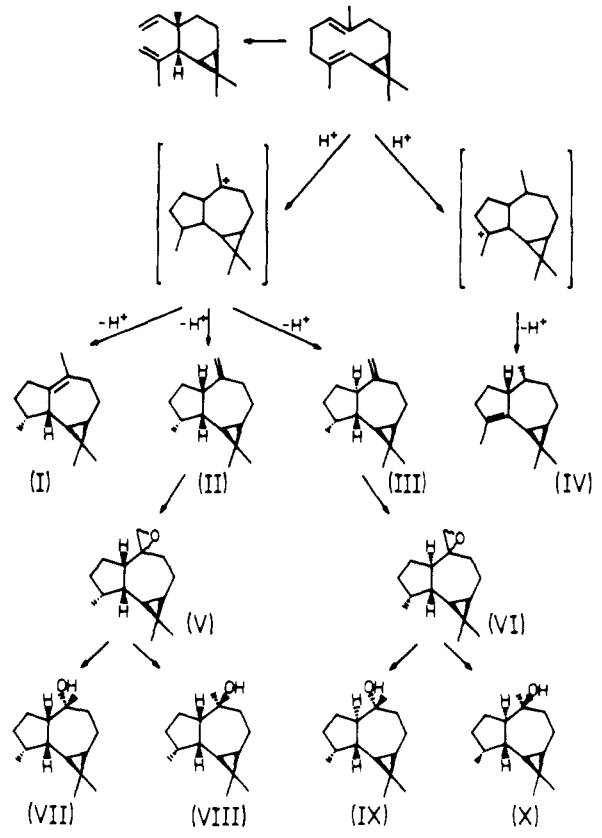

Figure 3. Possible reaction scheme to explain the transformation of bicyclogermacrene into tricyclic sesquiterpenes. Components identified in the hop variety Hersbrucker Spät: (I) = viridiflorene; (II) = alloaromadendrene; (III) aromadendrene; (IV) = α-gurjunene; (V) = alloaromadendrene epoxide; (VI) = aromadendrene epoxide; (VII) = ledol; (VIII) = viridiflorol; (IX) = globulol; (X) = epiglobulol.

cadinol, δ-cadinol, α-cadinol, humulenol II, and farnesol were also identified in the variety Northern Brewer. These components are common sesquiterpenoids in most hop varieties.

Aromadendrene epoxide and alloaromadendrene epoxide were identified for the first time in hop. The components were synthesized by reaction of the corresponding hydrocarbons with 3-chloroperbenzoic acid. The formed epoxides were reduced to the alcohols by treatment with $LiAlH_4$. Aromadendrene epoxide was transformed into globulol and epiglobulol. Both alcohols could be identified in the hop oil. In an analogous reaction alloaromadendrene epoxide formed ledol and viridiflorol.

The structures of the new hop components are presented in Figure 3. Cubenol (the transdecalin isomer of epicubenol) could be identified for the first time in hop. It is also present in the hop oil of Northern Brewer. Epoxidation of δ-cadinene formed two epoxides which were transformed (by reduction with $LiAlH_4$) into δ-cadinol, T-muurolol, epicubenol, and cubenol. In an analogous reaction γ-cadinene formed α-cadinol and T-cadinol. This reaction seems to be operative during the storage of hops when the concentrations of the cadinenetype alcohols increased considerably. Two epoxides were formed from β-selinene. β-Selinene epoxide I was determined as a minor constituent in the hop oil. The major part of the epoxide was transformed into selin-11-en-4-ol (Figure 4). The alcohol couuld be characterized for the first time in hop. 11,12-Epoxyselin-4-ene, which was not present in the hop oil, was transformed into β-eudesmol which is contained in high concentration in Hersbrucker Spät. In an analogous reaction selina-4,7-diene formed juniper camphor which was first characterized in Japanese hop varieties (Naya and Kotake, 1972). Compound 24 was formed

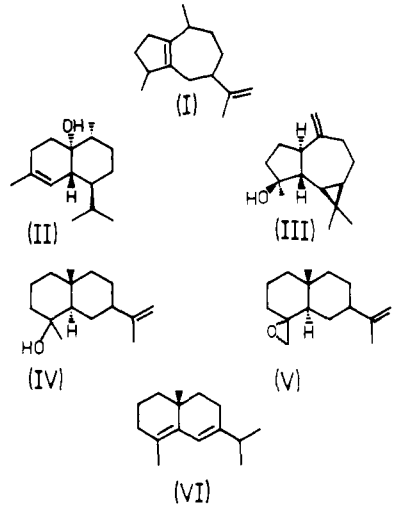

Figure 4. Structures of some components identified for the first time in the hop variety Hersbrucker Spät: (I) = α-guaiene; (II) = cubenol; (III) = spathulenol; (IV) = selin-11-en-4-ol; (V) = β-selinene epoxide I; (VI) = δ-selinene.

during epoxidation of alloaromadendrene and its mass spectrum is similar to that of palustrol (Stenhagen et al., 1974).

The tricyclic sesquiterpenes in hops seem to be formed from bicyclogermacrene which was identified by Nishimura et al. (1969) in the peel oil of *Citrus junos*. Aromadendrene was first identified in *Mentha piperita* by Vlahov et al. (1966) and aromadendrene and viridiflorene were identified in the essential oil of the Australian tea tree (Swords

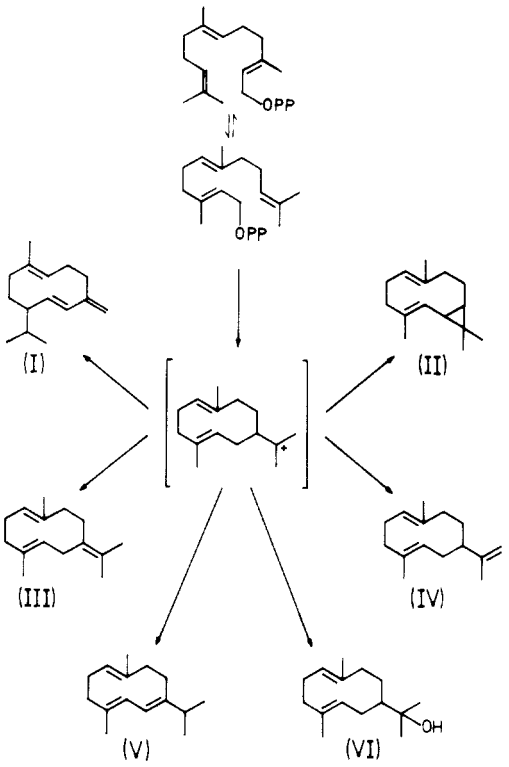

Figure 5. Simplified pathway to explain the formation of germacrenes in the hop variety Hersbrucker Spät: (I) = germacrene D; (II) = bicyclogermacrene; (III) = germacrene B; (IV) = germacrene A; (V) = germacrene C; (VI) = hedycaryol.

and Hunter, 1978). Germacrene B was identified in hop of the varieties Sunshine, Hersbruck Gebirg, Tasmanian White Vine, and Late Vine by Hartley and Fawcett (1969), and the authors supposed that this compound may be responsible for the resistance against powdery mildew.

Biogenesis of Sesquiterpenes in Hersbrucker Spät. The hop oil of Hersbrucker Spät contains humulene, β-caryophyllene, muurolenes, cadinenes, and the corresponding alcohols which are present in the most hop varieties. The biogenesis of these sesquiterpenes was investigated by biogenetic-type rearrangement studies as summarized by Coates (1976). (*E,E*)-Farnesyl-PP may be transformed into humulenes and caryophyllenes by a 1,10-cyclization. This pathway seems to be operative in all hop varieties. As shown in Figure 5, (*E,E*)-farnesyl-PP may be transformed via an (*E,E*)-1,5-decadienyl cation into germacrene D, germacrene B, and bicyclogermacrene which were characterized in Hersbrucker Spät. Hedycaryol and germacrene A may also be formed by this reaction. Germacrene D is a potent precursor of cadinenes and muurolenes as shown by Yoshihara et al. (1969) and discussed by Naya and Kotake (1972) for hop.

Figure 6 presents a possible scheme which may explain the transformation of germacrene B into γ-elemene (cope rearrangement) and into selinadienes by an electrophilic cyclization. Electrophiles effect regioselective addition to the 1,2 double bond and stereospecific cyclization to *trans*-decalins. During this reaction juniper camphor may be formed. In an analogous reaction hedycaryol may be transformed into α- and β-selinenes which were determined as strongly concentrated constituents in Hersbrucker Spät. The tricyclic sesquiterpenes α-gurjunene, aromadendrene, alloaromadendrene, and viridiflorene were identified for the first time as hop constituents. Bicyclogermacrene, first characterized by Nishimura et al. (1969) in the peel oil of *Citrus junos*, may act as a precursor of tricyclic sesquiterpenes and can be transformed into bicycloelemene via

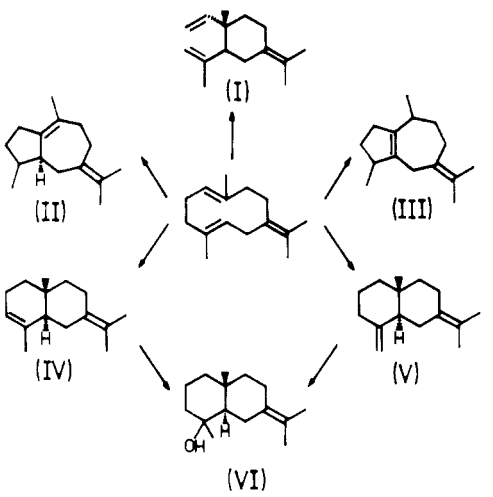

Figure 6. Scheme which may explain the transformation of germacrene B into other sesquiterpenes: (I) = γ-elemene; (II) = β-bulnesene; (III) = β-guaiene; (IV) = selina-3,7-diene; (V) = selina-4,7-diene; (VI) = juniper camphor.

cope rearrangement (Figure 3). Bicyclogermacrene is a key intermediate of aromadendrene, alloaromadendrene, and derivatives viridiflorene, and δ-cadinene. The tricyclic sesquiterpenes are also present in Hüller Bitterer, Perle, and Hallertauer Mittelfrüh as minor constituents. But they are not present in Northern Brewer, Brewer's Gold, Saazer, Spalt, and Tettnanger.

Registry No. α-Cubebene, 17699-14-8; α-ylangene, 14912-44-8; α-copaene, 3856-25-5; α-gurjunene, 489-40-7; β-cubebene, 13744-15-5; β-caryophyllene, 87-44-5; aromadendrene, 489-39-4; α-guaiene, 3691-12-1; γ-elemene, 30824-67-0; alloaromadendrene, 25246-27-9; β-farnesene, 18794-84-8; humulene, 6753-98-6; γ-muurolene, 24268-39-1; δ-selinene, 28624-28-4; viridiflorene, 21747-46-6; germacrene D, 23986-74-5; δ-guaiene, 3691-11-0; β-selinene, 17066-67-0; α-selinene, 473-13-2; α-muurolene, 17627-24-6; bicyclogermacrene, 24703-35-3; γ-cadinene, 483-74-9; δ-cadinene, 483-76-1; selina-4,7-diene, 515-17-3; selina-3,7-diene, 6813-21-4; α-cadinene, 24406-05-1; calamenene, 483-77-2; germacrene B, 15423-57-1; cadalene, 483-78-3; aromadendrene epoxide (isomer 1), 85710-39-0; aromadendrene epoxide (isomer 2), 85760-80-1; β-caryophyllene epoxide, 1139-30-6; humulene epoxide I, 19888-33-6; alloaromadendrene epoxide (isomer 1), 85760-81-2; alloaromadendrene epoxide (isomer 2), 85760-82-3; humulene epoxide II, 19888-34-7; β-selinene epoxide I (isomer 1), 37900-23-5; β-selinene epoxide I (isomer 2), 85760-83-4; epiglobuloe, 55659-76-2; ledol, 577-27-5; caryolan-1-ol, 472-97-9; globulol, 489-41-8; viridiflorol, 552-02-3; epicubenol, 19912-67-5; cubenol, 21284-22-0; spathulenol, 6750-60-3; γ-eudesmol, 1209-71-8; T-cadinol, 5937-11-1; δ-cadinol, 36564-42-8; α-eudesmol, 473-16-5; β-eudesmol, 473-15-4; α-cadinol, 481-34-5; selin-11-en-4-ol, 71963-78-5; humulenol II, 19888-00-7; juniper camphor, 473-04-1; palustrol, 5986-49-2; farnesol, 4602-84-0; 11,12-epoxyselin-4-ene, 85710-38-9; T-muurolol, 19912-67-5.

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Fractionation of Bright Tobacco

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Polymeric cell wall components were determined for ground, uncased bright tobacco lamina by using a systematic fractionation scheme. Starch was determined by an enzymatic procedure utilizing a thermophilic amylase. Analysis for pectin utilized an acid-catalyzed decarboxylation method. Lignin was determined by both the Klason lignin procedure and ^{13}C NMR integration of aromatic carbons. Estimation of protein was based on the results of Kjeldahl digestion. Hemicellulose and cellulose were determined by a capillary gas chromatography procedure for quantitation of neutral sugars. The results from this fractionation procedure were compared with those from a serial extraction procedure. Values for all components except cellulose were found to vary considerably between the two procedures. Errors in the serial extraction procedure were attributed to the fact that many distinct types of biopolymers have overlapping ranges of solubility. The cell walls of cured bright lamina were found to resemble the primary cell walls of dicots.

Tobacco has been one of the most thoroughly analyzed of all plant materials. However, tobacco cell wall biopolymers have received relatively little attention. In general, these compounds have been individually isolated from tobacco for analysis and characterization. Further, the analyses of these biopolymers have frequently utilized nonspecific gravimetric procedures which introduced potential errors since many distinct types of cell wall components have overlapping ranges of solubility. Thus, Bourne et al. (1967) obtained pectin from bright tobacco by ammonium oxalate extraction and Christy and Samfield (1960) isolated cellulose from various tobacco types by a 17-step procedure. Typically hemicellulose has not been isolated; instead, analyses have been performed for pentosans (Phillips and Bacot, 1953) which are found in pectin as well as hemicellulose. Over the past few years Katō and co-workers have characterized several biopolymers isolated from various tobacco materials (Eda et al., 1976, 1977; Eda and Katō, 1978, 1980; Mori et al., 1979, 1980; Mori and Katō, 1981). To date, no overall fractionation scheme to separate systematically the cell wall biopolymers prior to analysis has been presented.

Other plant materials, especially forage crops, have been analyzed by using more comprehensive fractionation schemes. Van Soest and colleagues have pioneered the use of detergent extraction procedures (Van Soest, 1963; Van Soest and Wine, 1967, 1968; Goering and Van Soest, 1970). More recently, procedures for the chemical characterization of water-soluble and water-insoluble dietary fibers have been developed (Theander and Åman, 1979; Salomonsson et al., 1980).

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The present research was undertaken to develop methods for the systematic fractionation and characterization of cell wall biopolymers in tobacco based on procedures that had been used with other plant materials.

EXPERIMENTAL SECTION

Fractionation Procedure. The tobacco used for this work was heavy, or bodied, flue-cured bright tobacco lamina from the upper mid-stalk position. This uncased tobacco was ground to pass a 20-mesh screen prior to extraction.

The fractionation procedure is schematically presented in Figure 1. In the first step the tobacco sample (100.0 g) was extracted with 80% ethanol in a Soxhlet apparatus for 18 h. The ethanol extraction solution was concentrated in vacuo to a constant weight in a tared round-bottom flask. The residue from the extraction was then dried overnight in a vacuum oven at 55 °C. For the removal of starch, the ethanol-extracted residue was added to 1200 mL of deionized water which had been preheated to 85 °C. The mixture was maintained at that temperature, with occasional swirling of the flask, for 30 min. Then 7.2 mL of Termamyl 60-L enzyme used as received from Novo Laboratories, Inc., was added, and the resultant mixture was heated for another 45 min at 85 °C with occasional swirling. Next, this mixture was brought to reflux and maintained at that temperature for 15 min. Finally, the mixture was filtered through a Büchner funnel with a sintered glass filter disk (ASTM 40-60). The filtrate was stored in a refrigerator. The residue was dried overnight in a vacuum oven at 55 °C.

The dried Termamyl-treated residue was refluxed under nitrogen for 3 h in a solution containing 2.5 L of 0.10 M aqueous KOH, 2.5 g of $NaBH_4$, and 1 mL of 1-octanol. After being refluxed, the mixture was allowed to cool to

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