Bound Aroma Compounds from the Fruit and the Leaves of Blackberry (*Rubus laciniata* L.)

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HRGC and HRGC-MS identifications of bound aroma compounds from the fruit and the leaves of blackberry (*Rubus laciniata* L.) were achieved after isolation of extracts obtained by Amberlite XAD-2 adsorption and methanol elution followed by hydrolysis with a commercial pectinase enzyme. In the fruit 41 aglycons and in the leaves 32 bound aroma compounds were identified. Quantitative HRGC revealed 5.8 mg/kg of aglycons in the fruit, whereas 56.1 mg/kg were determined in the leaves. Major constituents comprised benzyl alcohol, benzoic acid, 3-hydroxy-7,8-dihydro-β-ionol, (*Z*)-3-hexen-1-ol, and the diastereomeric vitispiranes. Chiral evaluation of enzymatically liberated aroma compounds carried out by on-line multidimensional capillary gas chromatography-mass spectrometry (MDGC-MS) revealed the occurrence of enantiomerically pure (*S*)-2-heptanol both in the fruits and in the leaves as well as 2-methyl-2-hepten-6-ol in the enantioselective distribution of *R*:*S* = 82:18% in the leaves.

INTRODUCTION

A few years ago, the aroma compounds of blackberry fruit (*Rubus laciniata* L.) were studied in detail (Georgilopoulos and Gallois, 1987a,b). The main constituent identified was 2-heptanol, followed by varying amounts of *p*-cymen-8-ol, 2-heptanone, 1-hexanol, α-terpineol, pulegone, 1-octanol, isoborneol, myrtenol, 4-terpinenol, carvone, elimicine, and 1-nonanol. In contrast to the composition of free volatiles, there is no information available about the bound aroma compounds in this fruit species. Due to the importance of the still unexploited potential of bound aroma substances in plant tissues, e.g., as flavor precursors (Williams et al., 1989), it was interesting to extend our knowledge of bound aroma compounds of plant origin (Williams et al., 1982, 1989; Günata et al., 1985; Strauss et al., 1987; Salles et al., 1988; Winterhalter and Schreier, 1988; Mayerl et al., 1989; Schwab et al., 1989, 1990; Winterhalter, 1990; Pabst et al., 1991; Wu et al., 1991; Krammer et al., 1991; Wintoch et al., 1991) to the study of the fruit and leaves of blackberry (*R. laciniata* L.). This paper concerns the results obtained during this investigation.

EXPERIMENTAL PROCEDURES

Solvents. All solvents employed were of high purity at purchase (Aldrich) and were redistilled before use.

Fruits. Fresh ripe fruits (*R. laciniata*, L. cv. Schwarze Perle) were obtained from the local market.

Isolation of a Glycosidic Extract. (*a*) *Fruits.* After homogenization of 2 kg of fruits with 1 L of 0.2 M phosphate buffer (pH 7.5) containing 0.2 M glucono-δ-lactone as glycosidase inhibitor and centrifugation (30 min; 10000*g*), the supernatant was subjected to LC chromatography on Amberlite XAD-2 adsorbent using a 25 × 900 mm glass column (Günata et al., 1985). After being washed with 1500 mL of H_2O and 500 mL of pentane, the extract was isolated by eluting with 1000 mL of MeOH. The MeOH fraction was concentrated under reduced pressure to dryness (rotavapor) and redissolved in 80 mL of 0.2 mL phosphate buffer (pH 5.5). Remaining volatiles were removed by diethyl ether extraction.

(*b*) *Leaves.* After mixing of 50 g of leaves with 300 mL of methanol and macerization of the mixture (adjusted to pH 7) at ambient temperature overnight, a clear extract was obtained by centrifugation (4000*g*; 30 min). Methanol was removed under reduced pressure (rotavapor); the aqueous residue was extracted with pentane-dichloromethane to separate free volatiles and chlorophyll and applied to an Amberlite XAD-2 column according to the procedure mentioned above under Fruits.

Enzymatic Hydrolysis. In a typical experiment, a nonselective pectinase (200 μL of Rohapect D5L; Röhm, Darmstadt) and a standard (60 μg of phenyl β-D-glucoside) were added to the glycosidic extract (a, 250 mg; b, 300 mg), and the mixture was incubated at 37 °C overnight. The liberated aglycons were extracted with diethyl ether, and the dried (anhydrous Na_2SO_4), filtered, and concentrated (Vigreux column, 45 °C) extract was subjected to HRGC and HRGC-MS analyses. In the same manner, blank tests without addition of enzyme were carried out.

Capillary Gas Chromatography (HRGC). A Carlo Erba Fractovap 4100 gas chromatograph with FID equipped with a J&W DB-Wax capillary column (30 m × 0.25 mm i.d., df = 0.25 μm) was used. Split injection (1:50) was employed. The temperature program was 3 min isothermal at 50 °C, increased at 4 °C/min to 240 °C and held at 240 °C for 15 min. The flow rate for the carrier gas was 1.6 mL/min He and for the makeup gas 30 mL/min N_2; for the detector gases the flow rates were 30 mL/min H_2 and 300 mL/min air. Injector and detector temperatures were kept at 250 °C. Volumes of 1 μL were injected.

Capillary Gas Chromatography-Mass Spectrometry (HRGC-MS). A Varian Aerograph 1440 gas chromatograph equipped with a Gerstel split injector (1:25) was connected by direct coupling to a Finnigan MAT 44 mass spectrometer. The same type of column as mentioned above for HRGC was used. The conditions were as follows: temperature program (DB-Wax), 3 min isothermal at 50 °C, raised from 50 to 220 °C at 4 °C/min and held at 220 °C for 20 min; carrier gas flow rate, 1.8 mL/min He; temperature of ion source and all connection parts, 200 °C; electron energy, 70 eV; cathodic current, 0.8 mA. Volumes of 1 μL were injected.

Results of qualitative analyses were verified by comparison of HRGC retention (R_i) and mass spectral data with those of authentic reference compounds.

Chiral Analysis. Chirality evaluation of 2-heptanol (as its acetate) and 2-methyl-2-hepten-6-ol was carried out by on-line coupled multidimensional capillary gas chromatography (DB-5/C-Dex B)-mass spectrometry (MDGC-MS) using a Siemens Sichromat 2 double-oven gas chromatograph with split injectors (250 °C; 1:38) and FIDs in oven 1 and oven 2. Preseparation in oven 1 was performed on a J&W DB-5 fused silica capillary column (30 m × 0.25 mm i.d.; df = 0.25 μm). The temperature program was 60-300 °C at 5 °C/min. Thirty s-cuts were carried out by a Siemens "live switching system" (Oreans et al., 1984) to a J&W C-Dex B fused silica capillary column (30 m × 0.25 mm i.d.) in oven 2. The conditions were as follows: temperature program, 20 min isothermal at 60 °C, raised from 60 to 220 °C at 2 °C/min; carrier gas flow rates, 0.65 and 0.89 mL/min He in ovens 1 and

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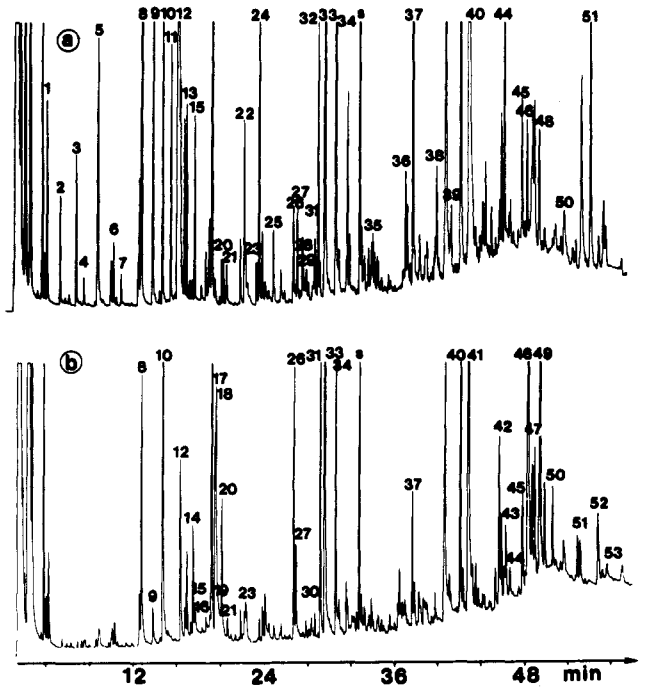

Figure 1. HRGC–MS separations of aglycons from the fruit (a) and the leaves (b) of blackberry on a J&W DB-Wax WCOT capillary column (30 m × 0.25 mm i.d.; df = 0.25 μm). The numbers correspond to the numbers outlined in Table I. s, standard.

2, respectively; detector gases, 39 mL/min H_2, 221 mL/min air and 39 mL/min H_2, 194 mL/min air in ovens 1 and 2, respectively. MDGC–MS coupling was achieved by a variable exit splitter (Siemens; 1:99; transfer line, 200 °C) directly to the ion source of the above-mentioned mass spectrometer.

Identifications were carried out by means of MDGC and MDGC–MS data of authentic racemic and optically enriched enantiomers of 2-heptyl acetate and 2-methyl-2-hepten-6-ol (cf. Reference Compounds).

Reference Compounds. Optically enriched (*R*)-2-heptyl acetate and (*R*)-2-methyl-2-hepten-6-ol were prepared by porcine pancreas lipase catalyzed esterification of the corresponding racemic alcohols as described previously (Gerlach et al., 1989).

RESULTS AND DISCUSSION

Typical HRGC–MS separations of the aglycons enzymatically released from glycosidic extracts isolated from the fruits and the leaves of blackberry (*R. laciniata* L.) are outlined in Figure 1. In Table I, the individual aglycons and their amounts determined are summarized. In total, in the fruit 41 and in the leaves 32 bound aroma compounds were identified. Quantitative HRGC revealed 5.8 mg/kg aglycons in the fruit, whereas 56.1 mg/kg was determined in the blackberry leaves. In contrast to the phenolic odor exhibited by the extract from the fruit, the odor of the leaf concentrate was sweet-flowery with green aspects.

Similarly to the results obtained in our recent studies on bound aroma compounds in fruits (Pabst et al., 1991; Krammer et al., 1991; Wintoch et al., 1991), the aglycons from blackberry fruit and leaves are mainly derived from fatty acid, phenylpropanoid, and terpene metabolism. In the group of glycosidically bound alcohols, 2-heptanol is of particular analytical interest. Previously, it has been found in enantiomerically pure *S* form (Engel, 1988) as the main constituent of free blackberry fruit volatiles (Georgilopoulos and Gallois, 1987a). Chiral evaluation of bound 2-heptanol from the fruit and the leaves also revealed the presence of the optically pure (100 % ee) *S* antipode. Among the chiral constituents detected in blackberry leaves (Table I), 2-methyl-2-hepten-6-ol, also known as sulcatol, the aggregation pheromone of the ambrosia beetles *Gnathotrichus sulcatus* and *G. retucus* (Borden et al., 1980), was determined to be present in the distinct enantiomeric distribution of *R*:*S* = 82:18%. Attempts to differentiate the enantiomers of vitispirane on the cyclodextrin phase used failed; only a separation of diastereomers was achieved. These latter compounds, being present with 2.6 mg/kg in the aglycon fraction of leaves, are obviously degradation products of an extreme labile nonvolatile precursor substance. Likely candidates

Table I. Compounds Identified in the Fruits and Leaves of Blackberry by HRGC and HRGC–MS after Enzymatic Hydrolysis (Rohapect D5L) of a Methanolic Eluate Obtained from XAD-Separated Fraction

peak no.[a]	R_i[b]	compound	amount,[c] mg/kg juice	leaves
1	1029	2-methyl-3-buten-1-ol	+	nd
2	1085	2-methyl-1-propanol	+	nd
3	1136	1-butanol	+	nd
4	1152	3-penten-2-ol	+	nd
5	1201	2-methyl-1-butanol	+	nd
6	1241	1-pentanol	+	nd
7	1270	3-hydroxy-2-butanone	+	nd
8	1318	2-heptanol	0.2	1.5
9	1343	1-hexanol	0.1	0.2
10	1370	(*Z*)-3-hexen-1-ol	+	4.1
11	1390	(*E*)-2-hexen-1-ol	+	nd
12	1424	acetic acid	0.3	1.0
13	1431	(*Z*)-linalool oxide, furanoid	+	nd
14	1451	2-methyl-2-hepten-6-ol	nd	0.6
15	1465	(*E*)-linalool oxide, furanoid	+	0.3
16	1487	theaspirane, isomer I	nd	+
17/18	1515	vitispirane, isomers I/II	nd	2.6
19	1522	theaspirane, isomer II	nd	+
20	1538	linalool	+	0.8
21	1550	1-octanol	+	0.2
22	1598	butanoic acid	+	nd
23	1600	*p*-menthen-9-al	+	0.2
24	1641	2-methylbutanoic acid	0.1	nd
25	1672	isoborneol	+	nd
26	1748	citronellol	+	0.9
27	1759	methyl 2-hydroxybenzoate	+	0.5
28	1773	myrtenol	+	nd
29	1782	nerol	+	nd
30	1810	damascenone	+	0.2
31	1830	geraniol	+	1.5
32	1836	guaiacol	+	nd
33	1852	benzyl alcohol	0.5	11.9
34	1890	2-phenylethanol	0.3	1.5
s	1965	phenol (standard)		
35	2022	3-phenyl-1-propanol	+	nd
36	2130	eugenol	+	nd
37	2160	4-vinylguaiacol	0.2	0.5
38	2252	cinnamic alcohol	+	nd
39	2312	vinylphenylcarbinol	+	nd
40	2358	4-vinylphenol	0.7	2.0
41	2387	benzoic acid	1.0	7.3
42	2525	3-hydroxy-β-damascone	nd	1.2
43	2550	3-hydroxy-7,8-dihydro-β-ionone	nd	0.7
44	2556	4-vinylsyringol	+	0.2
45	2625	3-oxo-α-ionol	+	0.5
46	2640	3-hydroxy-7,8-dihydro-β-ionol	+	7.3
47	2660	4-oxo-7,8-dihydro-β-ionol	nd	0.6
48	2694	3-oxo-7,8-dihydro-α-ionol	+	3.2
49	2715	3-hydroxy-7,8-dehydro-β-ionol	nd	1.4
50	2804	(*E*)-cinnamic acid	+	nd
51	2865	4-(4-hydroxy-3-methoxyphenyl)-1-butanol	nd	0.3
52	2932	vimifoliol	+	0.6
53	2970	7,8-dihydrovomifoliol	nd	0.2

[a] The peak numbers correspond to the numbers in Figure 1. [b] R_i, linear retention index based on a series of *n*-hydrocarbons. The R_i data given were coincident (∓5) with that of authentic reference compounds. For HRGC–MS conditions, see Experimental Procedures. [c] + = <0.1 mg/kg. nd, not detectable.

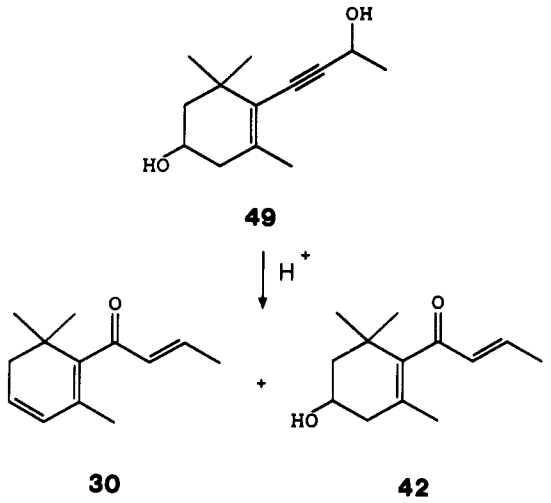

Figure 2. 3-Hydroxy-7,8-dihydro-β-ionol (**49**) as direct precursor of damascenone (**30**) and 3-hydroxy-β-damascone (**42**) (Sefton et al., 1989).

for precursors of these attractive aroma compounds have been presented in previous studies (Winterhalter and Schreier, 1988).

Among the aliphatic alcohols identified, the high amount of bound (*Z*)-3-hexen-1-ol in the leaves is striking. In general, C_6 alcohols are regarded to be catabolic products formed after cell disruption from *cis,cis*-1,4-pentadiene structured fatty acids by a cascade of enzymatic reactions (Hatanaka et al., 1986). The present findings indicate that anabolic pathways also have to be considered for the formation of C_6 volatiles. Glycosides of hexanol and (*Z*)-3-hexen-1-ol have already been identified in plant tissues (Schwab et al., 1988; Otsuka et al., 1990).

Besides a number of C_{13} norisoprenoids frequently occurring among bound aroma compounds of fruits (Winterhalter, 1990; Winterhalter and Schreier, 1988; Williams et al., 1989), the acetylenic diol **49** is of particular interest due to its important role as flavor precursor. Recently, it has been described to give damascenone (**30**) and 3-hydroxy-β-damascone (**42**) under acidic conditions (Sefton et al., 1989) (Figure 2). Thus, a glycosidic derivative of **49** could account at least in part for the damacenone generated after enzymatic treatment of the glycosidic extract from blackberry leaves.

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