JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Reinvestigation of the Bitter Compounds in Carrots (*Daucus carota* L.) by Using a Molecular Sensory Science Approach

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In order to reinvestigate the key molecules inducing bitter off-taste of carrots (*Daucus carota* L.), a sensory-guided fractionation approach was applied to bitter carrot extracts. Besides the previously reported bitter compounds, 6-methoxymellein (**1**), falcarindiol (**2**), falcarinol (**3**), and falcarindiol-3-acetate (**4**), the following compounds were identified for the first time as bitter compounds in carrots with low bitter recognition thresholds between 8 and 47 μmol/L: vaginatin (**5**), isovaginatin (**6**), 2-epilaserine oxide (**7**), laserine oxide (**8**), laserine (**14**), 2-epilaserine (**15**), 6,8-*O*-ditigloyl- (**9**), 6-*O*-angeloyl-, 8-*O*-tigloyl- (**10**), 6-*O*-tigloyl-, 8-*O*-angeloyl- (**11**), and 6-, 8-*O*-diangeloyl-6*ss*,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**12**), as well as 8-*O*-angeloyl-tovarol (**13**) and α-angeloyloxy-latifolone (**16**). Among these bitter molecules, compounds **9**, **10**, **13**, and **16** were not previously identified in carrots and compounds **6**, **11**, and **12** were yet not reported in the literature.

KEYWORDS: carrots; bitter taste; sensometabolome; sensometabolites; α-angeloyl-tovarol; laserine; laserine oxide; α-angeloyloxy-latifolone; epilaserine; epilaserine oxide

INTRODUCTION

Although modern breeding techniques and cultivar selection have been helpful to improve the sensory quality, carrots are able to produce a sporadic bitter off-taste when exposed to abiotic stress during harvesting, transportation, storage, and processing (*1*−*6*). In consequence, this sporadic bitter taste is often the reason for consumer rejection of carrots and carrot products such as juice and puree and is, therefore, a major problem for vegetable processors.

About 50 years ago, sensory studies revealed that bitter taste development occurred, in particular, in the phloem tissue of carrots when stored in the cold (*1*). In 1956, the observed correlation between the intensity of the absorption maximum at 265 nm of a petroleum ether extract and the bitter overall taste of the carrot tissue led to the identification of 3-methyl-6-methoxy-8-hydroxy-3,4-dihydroisocoumarin, more commonly known as 6-methoxymellein (**1**) (**Figure 1**), as a candidate bitter taste compound (*2*). However, on the basis of more recent sensory studies and quantitative studies, the content of 6-methoxymellein was concluded to have no significant contribution to the overall bitter taste of carrots (*3*−*6*).

Although various studies have investigated bitter taste of carrots in recent years, the key stimuli inducing that typical bitterness are rather contradictory. For example, a study on the influence of temperature and plant density on the sensory quality suggested volatile terpenes to contribute to the sensorial score for bitterness (*7*), whereas an investigation on the influence of ethylene on bitter taste development revealed that some abnormal phenols including 6-methoxymellein, 5,7-dihydroxy-2-methylchromone, and 5-hydroxy-7-methoxy-2-methylchromone are produced as bitter molecules, which were not present in carrot tissue stored in the presence of aerial oxygen (*8*). Other groups found correlations between the production of ethylene and the content of 6-methoxymellein, but not between 6-methoxymellein content and bitterness (*3*). In consequence, the

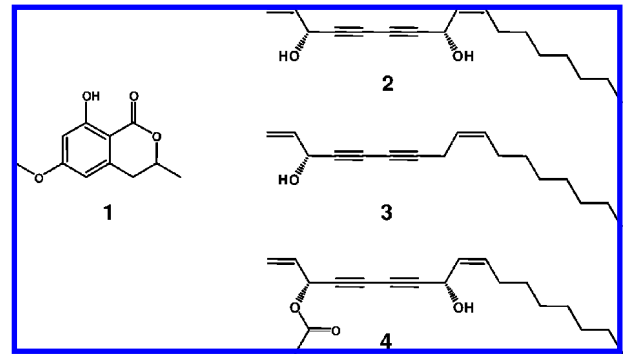

Figure 1. Chemical structures of bitter taste compounds previously reported in carrots: 6-methoxymellein (**1**), falcarindiol (**2**), falcarinol (**3**), and falcarindiol-3-acetate (**4**).

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10.1021/jf8023358 CCC: $40.75
Published on Web 10/16/2008

authors suggested that the bitterness might be due to additional, yet unknown, bitter molecules, the biosynthesis of which might be ethylene dependent. In a most recent study, a quantitative correlation between the contents of dicaffeoyl quinic acid and overall bitter taste of carrots was reported, but the authors did not prove any sensory contribution of this polyphenol to the bitter taste of carrots by means of spiking experiments (*6*).

Driven by the need to discover the key players imparting taste and/or off-taste of foods, the research area of "sensometabolomics" (*9*) has made tremendous efforts in recent years to identify, catalog, quantify, and evaluate the contribution of sensory active key metabolites to the taste of fresh and processed foods. Using the taste dilution analysis (TDA) (*10*) as a screening tool, multiple bitter tasting fractions were located in a solvent extract prepared from bitter carrots. Among these compounds, the bitter bisacetylenes falcarindiol (**2**), falcarinol (**3**), and falcarindiol-3-acetate (**4**) (**Figure 1**) were successfully identified, but the identity of other intense bitter fractions remained unknown (*4*). Although a quantitative study exhibited a good correlation between the content of falcarindiol (**2**) and the overall bitterness of multiple carrot samples (*5*), it was assumed that some of the unknown bitter compounds, previously located by means of the TDA approach (*4*), show a significant contribution to the bitter taste of carrots.

The objectives of the present investigation were, therefore, to screen for orphan bitter molecules in the sensometabolome of carrots by means of a molecular sensory science approach, and to determine the bitter recognition thresholds of these sensometabolites.

MATERIALS AND METHODS

Chemicals. The following compounds were obtained commercially: *n*-pentane, diethyl ether, ethanol, sodium chloride, lactic acid, monosodium L-glutamate, magnesium sulfate, salicin, caffeine (Merck, Darmstadt, Germany); solvents were of HPLC grade (Merck, Darmstadt, Germany). Samples of fresh carrots (*Daucus carota* L.) were purchased in local vegetable stores and were sensorially evaluated for bitter off-taste. The samples showing the strongest bitter off-taste were pooled and used for analysis. Reference material of 6-methoxymellein (**1**), falcarindiol (**2**), falcarinol (**3**), and falcarindiol-3-acetate (**4**) was isolated following the procedure reported recently (*4*).

Analytical Sensory Experiments. *General Conditions, Panel Training.* In order to familiarize the subjects with the taste language used by our sensory group and to get them trained in recognition and distinguishing different qualities of oral sensations in analytical sensory experiments, twelve assessors (seven women and five men, ages 23−29 years), who gave informed consent to participate in the sensory tests of the present investigations and had no history of known taste disorders, participated for at least two years in weekly training sessions. The subjects were trained to evaluate the taste of aqueous solutions (2 mL; pH 6.5) of the following standard taste compounds in bottled water (Evian, low mineralization, 500 mg/L): NaCl (20 mmol/L) for salty taste, lactose (50 mmol/L) for sweet taste, L-lactic acid (20 mmol/L) for sour taste, and monosodium L-glutamate (3 mmol/L) for umami taste. For training of bitter taste, solutions of $MgSO_4$ (166 mmol/L) representing a short-lasting metallic bitter taste quality perceived mainly at the anterior part of the tongue, salicin (1.4 mmol/L) imparting a long-lasting bitter taste sensation perceived mainly in the back of the tongue as well as the throat, and caffeine (17.0 mmol/L) providing a long-lasting bitterness perceived overall in the oral cavity were used as references. The sensory sessions were performed at 21 °C in an air-conditioned room with separated booths in three independent sessions. To prevent cross-modal interactions with odorants, the panelists used nose clips.

Precautions Taken for Sensory Analysis of Food Fractions and Taste Compounds. Prior to sensory analysis, solvent traces were removed from the freeze-dried fractions isolated from carrots. To achieve this, the individual fractions were dissolved in water and remaining volatiles and solvent traces were removed under high vacuum (<5 mPa, 35 °C). The individual residue was then again taken up in water and freeze-dried twice. HRGC−MS analysis and ion-chromatographic analysis revealed that food fractions treated with that procedure are essentially free of the solvents and buffer compounds used.

Table 1. Bitter Taste Intensity of Fractions Isolated from Carrots

fraction[a]	bitter taste intensity[b]	yield [mg/kg][c]
C	0	119400
B	1	1000
A	4.5	180[d]
A-I	0	3.0
A-II	0.7	57.4
A-III	3	23.9
A-IV	4.5	46.3
A-V	4	6.0

[a] Individual fractions contain the *n*-pentane extractables (fraction A), ethyl acetate extrables (fraction B), the nonsoluble residue (fraction C), as well as the fractions A-I to A-V isolated from the fraction A by means of silica gel chromatography. [b] Bitter intensity was evaluated on a scale of 0 (not detectable) to 5 (intense bitterness). [c] Yields were determined by weight. [d] The yield determined for this fraction might be somewhat too high as it was hygroscopic.

Determination of the Bitter Intensity of Fractions Isolated from Carrots. Aliquots of single fractions isolated from carrots were dissolved in their "natural" concentration ratios in bottled water (10.0 mL), which was adjusted to pH 6.5 with aqueous formic acid, and were then presented to the trained sensory panel who was asked to determine the bitter taste intensity by means of a duo test on a scale from zero (no bitter taste) to five (strong bitter taste) relative to a series of aqueous caffeine solutions in ascending concentrations 0.1 mmol/L (score 0), 1.5 mmol/L (score 1.0), 2.0 mmol/L (score 2.0), 2.5 mmol/L (score 3.0), 2.8 mmol/L (score 4.0) to 3,5 mmol/L (intensity 5.0).

Taste Recognition Threshold Concentrations. Threshold concentrations of purified bitter compounds were determined in bottled water adjusted to pH 6.5 with trace amounts of formic acid (1% in water) using triangle tests with ascending concentrations of the stimulus following the procedure reported previously (*11*). The threshold values of the sensory group was approximated by averaging the threshold value of the individuals in three independent sessions. Values between individuals and separate sessions differed by not more than ± one dilution step; that is, a threshold value of 20.0 μmol/L for 6-methoxymellein represents a range from 10.0 to 40.0 μmol/L.

Sensory-Directed Fractionation of Carrots. Carrots (10 kg) were minced in a blender and then sequentially extracted with *n*-pentane (4 × 7 L), followed by ethyl acetate (3 × 7 L) at room temperature under an atmosphere of argon. After filtration, the corresponding *n*-pentane isolates and ethyl acetate phases were combined and separated from solvent using vacuum to obtain the *n*-pentane extract (fraction A; yield: 1.8 g), the ethyl acetate extract (fraction B; yield: 10 g), as well as the nonsoluble residue (fraction C; yield: 1194 g) (**Table 1**). An aliquot (0.4 g) of the *n*-pentane extract was dissolved in a mixture (95/5, v/v; 5 mL) of *n*-pentane and diethyl ether and was then applied onto the top of a water-cooled 400 × 50 mm glass column filled with silica gel hydrated with 5% water. Chromatography was performed by eluting the column with mixtures (600 mL each) of *n*-pentane and diethyl ether in ratios (v/v) of 95/5 (fraction A-I), 80/20 (fraction A-II), 70/30 (fraction A-III), 50/50 (fraction A-IV), followed by diethyl ether (fraction A-V). Fractions A-IV and A-V were combined to give fraction A-IV/V and, together with the other fractions, were separated from solvent using vacuum to afford oily materials, which were kept at −20 °C for a maximum of 2 weeks until use for sensory studies and analytical experiments.

Isolation of Bitter Taste Compounds from Fractions A-III and A-IV/V. Aliquots of fraction A-III and A-IV/V, respectively, were dissolved in a mixture (70/30, v/v; 10 mL) of methanol and water, and, after membrane filtration, were fractionated by means of preparative HPLC on a Microsorb RP-18, 250 × 21.2 mm i.d., 5 μm column (Varian, Darmstadt, Germany) using a methanol/water gradient at a flow rate of 18 mL/min. Using water as solvent A and methanol as

Table 2. Bitter Taste Intensities of Fractions A-IV/V/1−A-/IV/V/14

fraction[a]	bitter taste intensity[b]	bitter compound identified[c]
A-IV/V/1	3.5	**1**
A-IV/V/2	2	
A-IV/V/3	3	**8**
A-IV/V/4	4	**7**
A-IV/V/5	4	**6**
A-IV/V/6	4.5	**5**
A-IV/V/7	5	**2**
A-IV/V/8	2.5	**9**
A-IV/V/9	2.5	**10**
A-IV/V/10	2.5	**11**
A-IV/V/11	2.5	**12**
A-IV/V/12	0.5	
A-IV/V/13	0	
A-IV/V/14	0	

[a] Number of HPLC-fraction referring to **Figure 2A**. [b] Intensity rated on a scale of 0 to 5 (0, no taste; 5, intensive bitter taste) relative to a series of aqueous caffeine solutions starting from 0.1 (intensity 0) to 32 mmol/L (intensity 5.0). [c] The structures of the compounds given as numbers are displayed in **Figures 1** and **3**.

Table 3. Bitter Taste Intensities of Fractions A-III/1−A-III/14

fraction[a]	bitter taste intensity[b]	bitter compounds identified[c]
A-III/1	5	**1**
A-III/2	2.5	
A-III/3	2.5	**16**
A-III/4	3.5	
A-III/5	3.5	
A-III/6	1.5	**14**
A-III/7	3.5	**15**
A-III/8	5	
A-III/9	2.5	**4**
A-III/10	0	
A-III/11	1	
A-III/12	5	**13**
A-III/13	2	**3**
A-III/14	2	

[a] Number of HPLC-fraction referring to **Figure 2B**. [b] Intensity rated on a scale of 0 to 5 (0, no taste; 5, intensive bitter taste) relative to a series of aqueous caffeine solutions starting from 0.1 (intensity 0) to 32 mmol/L (intensity 5.0). [c] The structures of the compounds given as numbers are displayed in **Figures 1** and **3**.

solvent B, chromatography was performed using a linear gradient from 70 to 100% solvent B within 50 min, and thereafter was kept at 100% solvent B for 10 min. Individual HPLC fractions were collected from fraction A-III and A-IV/V, respectively, separated from solvent using vacuum, freeze-dried twice, and then used to evaluate their bitter intensities in aqueous solution. The compounds inducing the bitter taste in the HPLC fractions judged with the highest sensory impacts were isolated and purified by means of rechromatography using the same chromatographic conditions as detailed above. As checked by HPLC-ELSD as well as ^{1}H NMR analysis, the single bitter compounds were obtained with a purity of more than 98%. The structure of the bitter compounds **5**−**16** isolated from the individual HPLC fractions (**Tables 2** and **3**) were determined by means of LC−MS and 1D/2D-NMR spectroscopic experiments.

Vaginatin (**5**)*, Figure 3.* Colorless oil. UV/vis (MeOH): λ_{max} = 204, 216, 252, 288 nm. LC−MS (ESI$^+$): *m/z* 357.4 (100, $[M + Na]^+$), 352.4 (89, $[M + NH_4]^+$). ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ 6.02 (1H, dq, J = 1.5, 7.1 Hz, H-3′), 5.67 (1H, d, J = 7.5 Hz, H-5), 5.24 (1H, d, J = 7.5 Hz, H-4), 2.35−2.45 (3H, m, H-2β, H-7β, H-8β), 2.20−2.32 (2H, m, H-1, H-2α), 2.01−2.12 (3H, m, H-7α, H-8α, H-9), 1.96 (3H, m, J = 7.1, 1.5 Hz, H-4′), 1.83 (3H, m, J = 1.5 Hz, H-5′), 1.75 (3H, s, H-13), 1.05 (6H, m, H-11, H-12), 0.98 (3H, d, J = 6.8 Hz, H-10). ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): δ 217.6 (C-3), 166.1 (C-1′), 146.2 (C-6), 139.1 (C-3′), 127.1 (C-2′), 119.7 (C-5), 82.5 (C-8a, C-3a), 75.8 (C-4), 50.7 (C-1), 38.1 (C-2), 37.2 (C-8), 28.9 (C-7), 26.3 (C-9), 25.9 (C-13), 24.4 (C-11), 21.0 (C-10), 20.7 (C-5′), 18.4 (C-12), 15.7 (C-4′).

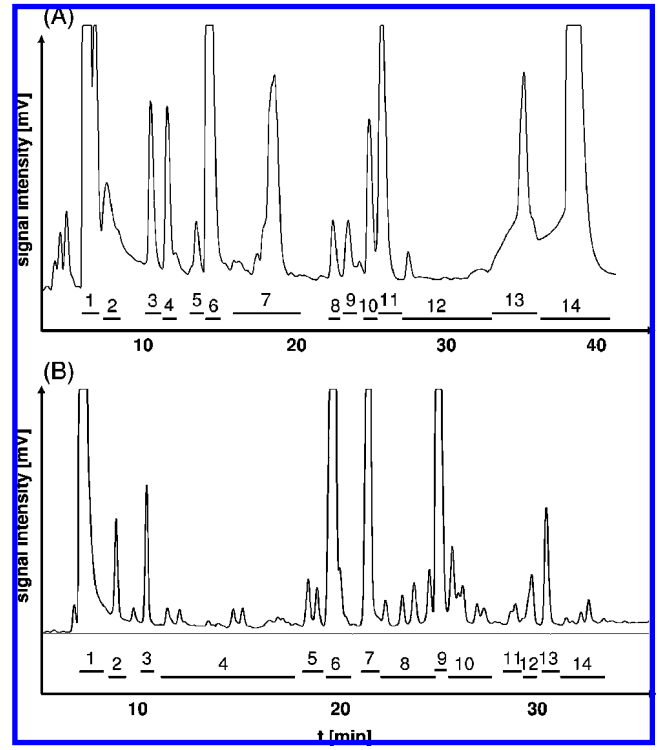

Figure 2. RP-HPLC chromatograms (200 nm) of fractions A-IV/V (**A**) and A-III (**B**), respectively.

Isovaginatin (**6**)*, Figure 3.* Colorless oil. UV/vis (MeOH): λ_{max} = 204, 216, 252, 288 nm. LC−MS (ESI$^+$): *m/z* 357.3 (100, $[M + Na]^+$), 352.4 (71, $[M + NH_4]^+$). LC−TOF-MS: *m/z* 357.2042 ($[M + Na]^+$, measured), *m/z* 357.2036 ($[M + Na]^+$, calcd for $C_{20}H_{30}O_4Na$). ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ 6.74 (1H, dq, J = 1.5, 7.1 Hz, H-3′), 5.67 (1H, d, J = 7.5 Hz, H-5), 5.24 (1H, d, J = 7.5 Hz, H-4), 2.35−2.45 (3H, m, H-2β, H-7β, H-8β), 2.20−2.32 (2H, m, H-1, H-2α), 2.01−2.12 (3H, m, H-7α, H-8α, H-9), 1.79 (3H, m, J = 7.1, 1.5 Hz, H-4′), 1.77 (3H, m, J = 1.5 Hz, H-5′), 1.75 (3H, s, H-13), 1.08 (3H, m, H-12), 1.05 (3H, m, H-11), 0.98 (3H, d, J = 6.8 Hz, H-10). ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): δ 217.6 (C-3), 166.1 (C-1′), 146.2 (C-6), 139.6 (C-3′), 127.1 (C-2′), 119.7 (C-5), 82.5 (C-8a, C-3a), 75.8 (C-4), 50.5 (C-1), 38.1 (C-2), 37.1 (C-8), 28.6 (C-7), 26.1 (C-9), 25.7 (C-13), 24.4 (C-11), 20.8 (C-10), 17.8 (C-12), 13.9 (C-5′), 11.9 (C-4′).

2-Epilaserinoxide (**7**)*, Figure 3.* Colorless oil. UV/vis (MeOH): λ_{max} = 212, 248, 284 nm. LC−MS (ESI$^+$): *m/z* 445.4 (4, $[M + K]^+$), 429.4 (45, $[M + Na]^+$), 424.5 (100, $[M + NH_4]^+$). ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ 6.58 (2H, s, H-2′, H-6′), 6.13 (1H, dq, J = 1.5, 7.1 Hz, H-3″), 5.96 (2H, s, H-7′), 5.86 (1H, d, J = 5.1 Hz, H-1), 5.29 (1H, dq, J = 5.1, 6.3 Hz, H-2), 3.90 (3H, s, H-8′), 3.00 (1H, q, J = 5.3 Hz, H-3‴), 2.02 (3H, dd, J = 7.1, 1.5 Hz, H-4″), 1.94 (3H, m, J = 1.5 Hz, H-5″), 1.47 (3H, s, H-5‴), 1.26 (3H, d, J = 6.3 Hz, H-3), 1.24 (3H, d, J = 5.3 Hz, H-4‴). ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): δ 169.0 (C-1‴), 166.0 (C-1″), 149.1 (C-5′), 143.6 (C-3′), 139.7 (C-3″), 135.8 (C-4′), 131.0 (C-1′), 127.6 (C-2″), 107.1 (C-6′), 101.6 (C-7′), 101.3 (C-2′), 76.0 (C-1), 72.7 (C-2), 59.8 (C-2‴, C-3‴), 56.6 (C-8′), 20.5 (C-5″), 19.0 (C-5‴), 15.5 (C-4″, C-3), 13.4 (C-4‴).

Laserinoxide (**8**)*, Figure 3.* Colorless oil. UV/vis (MeOH): λ_{max} = 212, 248, 284 nm. LC−MS (ESI$^+$): *m/z* 445.4 (4, $[M + K]^+$), 429.4 (45, $[M + Na]^+$), 424.5 (100, $[M + NH_4]^+$). ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ 6.56 (2H, s, H-2′, H-6′), 6.12 (1H, dq, J = 1.5, 7.1 Hz, H-3″), 5.96 (2H, s, H-7′), 5.73 (1H, d, J = 7.1 Hz, H-1), 5.34 (1H, m, J = 7.1, 6.3 Hz, H-2), 3.84 (3H, s, H-8′), 3.00 (1H, q, J = 5.3 Hz, H-3‴), 2.01 (3H, dd, J = 7.1, 1.5 Hz, H-4″), 1.90 (3H, m, J = 1.5 Hz, H-5″), 1.40 (3H, s, H-5‴), 1.23 (3H, d, J = 5.3 Hz, H-4‴) 1.18 (3H, d, J = 6.3 Hz, H-3). ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): δ 169.0 (C-1‴), 166.0 (C-1″), 149.1 (C-5′), 143.6 (C-3′), 139.7 (C-3″), 135.8 (C-4′), 131.0 (C-1′), 127.6 (C-2″), 107.1 (C-6′), 101.6 (C-7′), 101.3 (C-2′), 76.0 (C-1), 72.7 (C-2), 59.8 (C-2‴, C-3‴), 56.6 (C-8′), 20.5 (C-5″), 19.0 (C-5‴), 15.5 (C-4″, C-3), 13.4 (C-4‴).

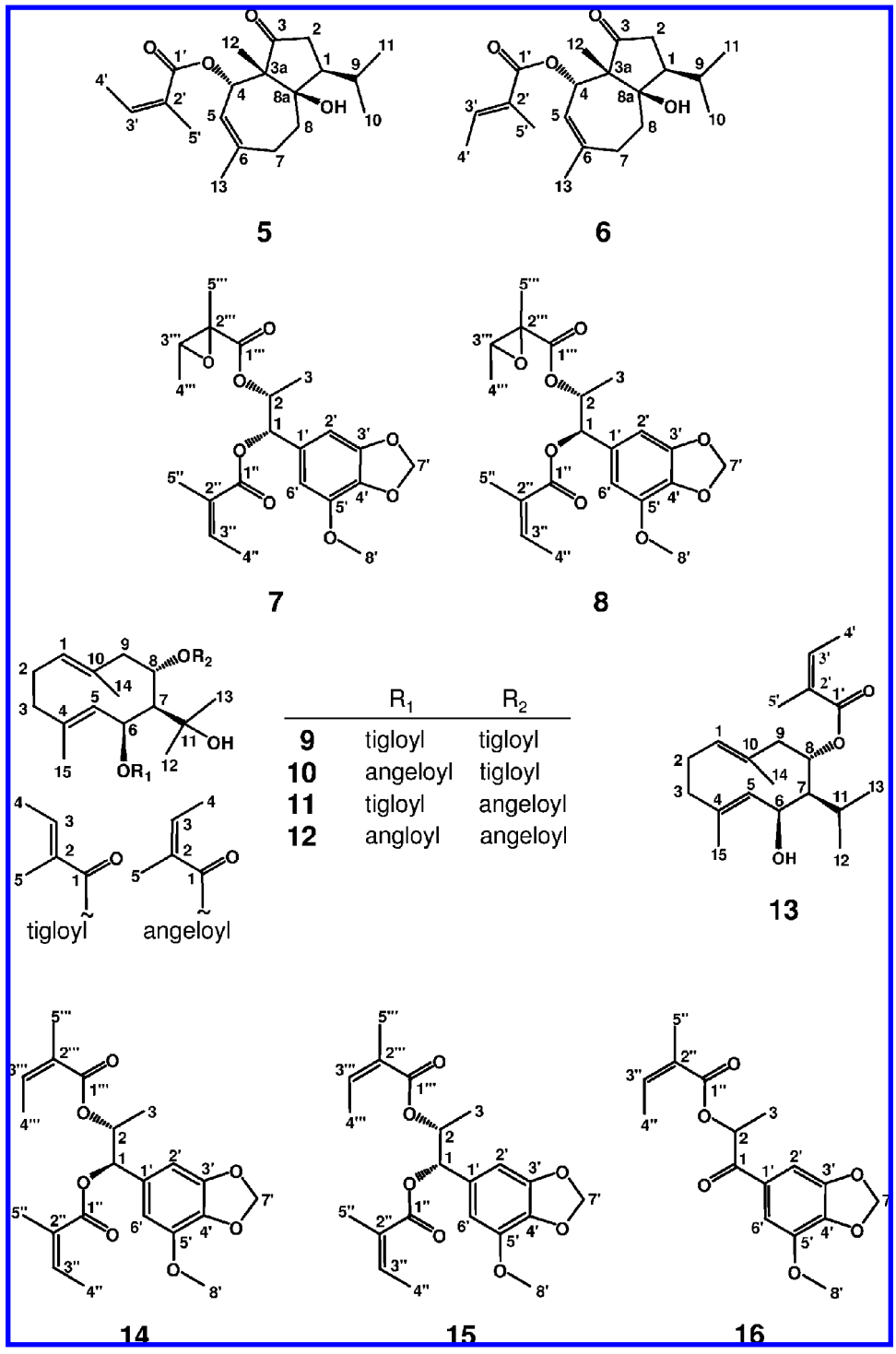

Figure 3. Chemical structures of the novel bitter taste compounds **5**−**16** identified in fractions A-III and A-IV/V of carrots: vaginatin (**5**), isovaginatin (**6**), 2-epilaserine oxide (**7**), laserine oxide (**8**), 6,8-*O*-ditigloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**9**), 6-*O*-angeloyl-8-*O*-tigloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**10**), 6-*O*-tigloyl-8-*O*-angeloyl- 6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**11**), 6,8-*O*-diangeloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**12**), 8-*O*-angeloyl-tovarol (**13**), laserine (**14**), 2-epilaserine (**15**), α-angeloyloxy-latifolone (**16**).

*6-O,8-O-Ditigloyl-6β,8α,11-trihydroxygermacra-1(10)E,4E-diene (**9**), Figure 3.* Colorless oil. UV/vis (MeOH): λ_{max} = 212 nm; LC−MS (ESI$^+$): *m/z* 441 (100, [M + Na]$^+$). ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ 6.87 (1H, dq, J = 1.5; 7.3 Hz, H-3″), 6.67 (1H, dq, J = 1.5, 7.3 Hz, H-3′), 5,80 (1H, d, J = 6.3 Hz, H-6), 5.67 (1H, dd, J = 5.6, 11.4 Hz, H-8), 5.17 (1H, m, H-1), 5.14 (1H, m, H-5), 2.79 (1H, dd, J = 5.8, 12.8 Hz, H_A-9), 2.42 (1H, m, H_A-2), 2.18 (2H, m, H-3), 2.14 (1H, m, H_B-2), 1.99 (1H, m, H_B-9), 1.90 (1H, m, H-7), 1.89 (3H, m, H-5″), 1.85 (3H, m, H-4″),1.70 (3H, m, H-5′), 1.69 (6H, m, H-14, H-4′), 1.56 (3H, m, H-15), 1.41 (3H, s, H-13), 1.38 (3H, s, H-12). ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): δ 166.8 (C-1′), 166.3 (C-1″), 137.6 (C-3″), 137.4 (C-3′), 135.4 (C-4), 132.0 (C-1), 130.4 (C-5), 128.9 (C-10), 129.0 (C-2′), 128.4 (C-2″), 74.6 (C-8), 73.2 (C-11), 70.4 (C-6), 52.9 (C-7), 40.9 (C-9), 38.5 (C-3), 30.3 (C-13), 29.1 (C-12), 24.8 (C-2), 20.6 (C-14), 16.8 (C-15), 14.5 (C-4″), 14.3 (C-4′), 12.1 (C-5″), 11.9 (C-5′).

*6-O-Angeloyl-8-O-tigloyl-6β,8α,11-trihydroxygermacra-1(10)E,4E-diene (**10**), Figure 3.* Colorless oil. UV/vis (MeOH): λ_{max} = 212 nm. LC−MS (ESI$^+$): *m/z* 441 (100, [M + Na]$^+$), 436 (40, [M + NH_4]$^+$). ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ 6.83 (1H, dq, J = 1.5; 7.3 Hz, H-3″), 6.02 (1H, dq, J = 1.5, 7.3 Hz, H-3′), 5,80 (1H, d, J = 6.3 Hz, H-6), 5.67 (1H, dd, J = 5.6, 11.4 Hz, H-8), 5.17 (1H, m, H-1), 5.14

(1H, m, H-5), 2.79 (1H, dd, J = 5.8, 12.8 Hz, H_A-9), 2.42 (1H, m, H_A-2), 2.18 (2H, m, H-3), 2.14 (1H, m, H_B-2), 1.99 (1H, m, H_B-9), 1.95 (3H, m, H-4′), 1.88 (1H, m, H-7), 1.86 (3H, m, H-5″), 1.81 (3H, m, H-4″),1.70 (3H, m, H-5′), 1.69 (3H, s, H-14), 1.56 (3H, m, H-15), 1.45 (3H, s, H-13), 1.40 (3H, s, H-12). ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): δ 166.8 (C-1′), 166.3 (C-1″), 139.5 (C-3′), 137.6(C-3″); 135.4 (C-4), 131.7 (C-1), 130.4 (C-5), 129.7 (C-10), 128.4 (C-2″), 127.2 (C-2′), 75.1 (C-8), 73.2 (C-11), 70.4 (C-6), 52.9 (C-7), 40.9 (C-9), 38.5 (C-3), 30.3 (C-13), 29.1 (C-12), 24.8 (C-2), 20.4 (C-5′, C-14), 16.8 (C-15), 15.6 (C-4′), 14.5 (C-4″), 12.3 (C-5″).

*6-O-Tigloyl-8-O-angeloyl-6β,8α,11-trihydroxygermacra-1(10)E,4E-diene (***11***), Figure 3.* Colorless oil. UV/vis (MeOH): λ_{max} = 212 nm; LC−MS (ESI^+): *m/z* 457 (86, $[M + K]^+$), 441 (100, $[M + Na]^+$), 436 (41, $[M + NH_4]^+$), 419 (15, $[M + H]^+$). LC−TOF-MS: *m/z* 441.2611 ($[M + Na]^+$, measured), *m/z* 441.2617 ($[M + Na]^+$, calcd. for $C_{25}H_{38}O_5Na$); ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ 6.70 (1H, dq, J = 1.5, 7.3 Hz, H-3′), 6.10 (1H, dq, J = 1.5; 7.3 Hz, H-3″), 5.80 (1H, d, J = 6.3 Hz, H-6), 5.67 (1H, dd, J = 5.6, 11.4 Hz, H-8), 5.17 (1H, m, H-1), 5.14 (1H, m, H-5), 2.79 (1H, dd, J = 5.8, 12.8 Hz, H_A-9), 2.42 (1H, m, H_A-2), 2.18 (2H, m, H-3), 2.14 (1H, m, H_B-2), 2.02 (3H, m, H-4″), 1.99 (1H, m, H_B-9), 1.91 (3H, m, H-5″), 1.90 (1H, m, H-7), 1.73 (3H, m, H-5′), 1.71 (3H, m, H-4′), 1.69 (3H, s, H-14), 1.56 (3H, m, H-15), 1.41 (3H, s, H-13), 1.38 (3H, s, H-12). ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): δ 167.1 (C-1′), 165.9 (C-1″), 139.6 (C-3″); 137.4 (C-3′), 135.4 (C-4), 132.0 (C-1), 130.4 (C-5), 128.9 (C-10), 128.8 (C-2′), 126.7 (C-2″), 74.6 (C-8), 73.2 (C-11), 70.4 (C-6), 52.9 (C-7), 40.9 (C-9), 38.5 (C-3), 30.3 (C-13), 29.1 (C-12), 24.8 (C-2), 20.6 (C-14, C-5″), 16.8 (C-15), 15.6 (C-4″) 14.3 (C-4′), 11.9 (C-5′).

*6-O-,8-O-Diangeloyl-6β,8α,11-trihydroxygermacra-1(10)E,4E-diene (***12***), Figure 3.* Colorless oil. UV/vis (MeOH): λ_{max} = 212 nm; LC−MS (ESI^+): *m/z* 457 (86, $[M + K]^+$), 441 (100, $[M + Na]^+$), 436 (41, $[M + NH_4]^+$), 419 (15, $[M + H]^+$); LC−TOF-MS: *m/z* 441.2615 ($[M + Na]^+$, measured), *m/z* 441.2617 ($[M + Na]^+$, calcd. for $C_{25}H_{38}O_5Na$). ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ 6.10 (1H, dq, J = 1.5; 7.3 Hz, H-3″), 6.02 (1H, dq, J = 1.5, 7.3 Hz, H-3′), 5,80 (1H, d, J = 6.3 Hz, H-6), 5.67 (1H, dd, J = 5.6, 11.4 Hz, H-8), 5.17 (1H, m, H-1), 5.14 (1H, m, H-5), 2.79 (1H, dd, J = 5.8, 12.8 Hz, H_A-9), 2.42 (1H, m, H_A-2), 2.18 (2H, m, H-3), 2.14 (1H, m, H_B-2), 2.02 (3H, m, H-4″), 1.99 (1H, m, H_B-9), 1.95 (3H, m, H-4′), 1.91 (3H, m, H-5″), 1.90 (1H, m, H-7), 1.74 (3H, m, H-5′), 1.69 (3H, s, H-14), 1.56 (3H, m, H-15), 1.41 (3H, s, H-13), 1.38 (3H, s, H-12). ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): δ 166.7 (C-1′), 165.9 (C-1″), 139.6 (C-3″); 139.4 (C-3′), 135.4 (C-4), 132.0 (C-1), 130.4 (C-5), 128.9 (C-10), 127.1 (C-2′), 126.7 (C-2″), 74.6 (C-8), 73.2 (C-11), 70.4 (C-6), 52.9 (C-7), 40.9 (C-9), 38.5 (C-3), 30.3 (C-13), 29.1 (C-12), 24.8 (C-2), 20.6 (C-14), 20.1 (C-5′), 16.8 (C-15), 15.6 (C-4′, C-4″).

*8-O-Angeloyl-tovarol (***13***), Figure 3.* Colorless oil. UV/vis (MeOH): λ_{max} < 200 nm. LC−MS (ESI^+): *m/z* 343 (100, $[M + Na]^+$). LC−TOF-MS: *m/z* 343.2107 ($[M + Na]^+$, measured), *m/z* 343.2244 ($[M + Na]^+$, calcd for $C_{20}H_{32}O_3Na$). ^{1}H NMR (800 MHz, $CDCl_3$, COSY): δ 6.17 (1H, dq, J = 1.5; 7.3 Hz, H-3′), 5.24 (1H, ddd, J = 11.0, 6.6, 1.6 Hz, H-8), 5.06 (1H, m, H-1), 4.86 (1H, m, H-5), 4.49 (1H, m, H-6), 2.63 (1H, dd, J = 6.2, 13.2 Hz, H_A-9), 2.39 (1H, m, H_A-2), 2.20 (2H, m, H_A-3), 2.13 (1H, m, H_B-2), 2.11 (1H, m, H_B-3), 2.09 (1H, m, H_B-9), 2.04 (3H, m, H-4′), 1.91 (3H, m, H-5′), 1.69 (3H, s, H-14), 1.65 (1H, m, H-11), 1.47 (3H, s, H-15), 1.33 (1H, m, H-7), 1.09 (3H, d, H-13), 1.05 (3H, d, H-12). ^{13}C NMR (200 MHz, $CDCl_3$, HMQC, HMBC): δ 168.9 (C-1′), 140.0 (C-3′); 133.3 (C-4, C-5), 131.7 (C-1), 128.9 (C-10), 126.7 (C-2′), 74.2 (C-8), 67.3 (C-6), 54.4 (C-7), 42.0 (C-9), 38.7 (C-3), 26.3 (C-11), 24.5 (C-2), 23.2 (C-13) 21.0 (C-12), 20.8 (C-14), 20.2 (C-5′), 16.0 (C-15), 15.8 (C-4′).

*Laserine (***14***),* **Figure 3.** Colorless oil. UV/vis (MeOH): λ_{max} = 216, 248, 272 nm. LC−MS (ESI^+): *m/z* 429.6 (64, $[M + K]^+$), 413.5 (100, $[M + Na]^+$), 408.5 (35, $[M + NH_4]^+$). ^{1}H NMR (400 MHz, pyridine-d_5, COSY): δ 6.87 (2H, m, H-2′, H-6′), 6.18 (1H, d, J = 7.1 Hz, H-1), 5.93−6.02 (4H, m, H-7′, H-3″, H-3‴), 5.57 (1H, m, H-2), 3.77 (3H, s, H-8′), 1.98−2.02 (6H, m, H-4″, H-4‴), 1.90−1.93 (6H, m, J = 1.5 Hz, H-5″, H-5‴), 1.23 (3H, d, J = 6.6 Hz, H-3). ^{13}C NMR (100 MHz, pyridine-d_5, HMQC, HMBC): δ 166.8 (C-1‴), 166.1 (C-1″), 149.4 (C-4′), 143.8 (C-5′), 139.2 (C-3″), 137.6 (C-3‴), 135.4 (C-3′), 132.3 (C-1′), 127.8 (C-2″, C-2‴), 108.1 (C-6′), 101.7 (C-7′), 101.5 (C-2′), 76.9 (C-1), 71.4 (C-2), 56.3 (C-8′), 20.3 (C-5″, C-5‴), 16.5 (C-3), 15.6 (C-4″, C-4‴).

*2-Epilaserine (***15***),* **Figure 3.** Colorless oil. UV/vis (MeOH): λ_{max} = 212, 248, 272 nm. LC−MS (ESI^+): *m/z* 429.5 (17, $[M + K]^+$), 413.3 (100, $[M + Na]^+$), 408.4 (26, $[M + NH_4]^+$). ^{1}H NMR (400 MHz, pyridine-d_5, COSY): δ 6.87 (2H, m, H-2′, H-6′), 6.33 (1H, d, J = 4.3 Hz, H-1), 6.02 (1H, dq, J = 1.5, 7.3 Hz, H-3″), 5.97 (2H, m, H-7′), 5.94 (1H, dq, J = 1.5, 7.3 Hz, H-3‴), 5.57 (1H, m, J = 4.3, 6.6 Hz, H-2), 3.78 (3H, s, H-8′), 2.02 (3H, m, J = 1.5, 7.3 Hz, H-4″), 1.97 (3H, m, J = 1.5, 7.3 Hz, H-4‴), 1.96 (3H, m, J = 1.5 Hz, H-5″), 1.89 (3H, m, J = 1.5 Hz, H-5‴), 1.32 (3H, d, J = 6.6 Hz, H-3). ^{13}C NMR (100 MHz, pyridine-d_5, HMQC, HMBC): δ 166.8 (C-1‴), 166.1 (C-1″), 149.4 (C-4′), 143.8 (C-5′), 139.2 (C-3″), 138.2 (C-3‴), 135.4 (C-3′), 131.9 (C-1′), 127.6 (C-2″, C-2‴), 107.7 (C-6′), 101.6 (C-7′), 101.1 (C-2′), 75.7 (C-1), 71.7 (C-2), 56.3 (C-8′), 20.3 (C-5″, C-5‴), 15.6 (C-4″, C-4‴), 14.8 (C-3).

*(Z)-1-(4-Methoxybenzo[d][1,3]dioxol-6-yl)-1-oxopropan-2-yl 2-methylbut-2-enoate (α-angeloyloxy-latifolone) (***16***),* **Figure 3.** Colorless oil;.UV/vis (MeOH): λ_{max} = 215, 305 nm. LC−MS (ESI^+): *m/z* 307.0 (100, $[M + H]^+$), 323.2 (62, $[M + NH_4]^+$), 328.9 (22, $[M + Na]^+$). LC−TOF-MS: *m/z* 329.0996 ($[M + Na]^+$, measured), *m/z* 329.0996 ($[M + Na]^+$, calcd for $C_{16}H_{18}O_5Na$). ^{1}H NMR (400 MHz, $CDCl_3$, COSY): δ 7.22 (1H, d, J = 1.5 Hz, H-6′), 7.09 (1H, d, J = 1.5 Hz, H-2′), 6.07 (1H, dq, J = 1.5, 7.3 Hz, H-3″), 6.00 (2H, s, H-7′), 5.86 (1H, q, J = 7.1 Hz, H-2), 3.87 (3H, s, H-8′), 1.94 (3H, dq, J = 1.5, 7.3 Hz, H-4″), 1.87 (3H, m, J = 1.5 Hz, H-5″), 1.49 (3H, d, J = 6.8 Hz, H-3). ^{13}C NMR (100 MHz, $CDCl_3$, HMQC, HMBC): δ 195.1 (C-1), 167.4 (C-1″), 149.0 (C-3′), 143.8 (C-5′), 139.9 (C-4′, C-1′), 139.3 (C-3″), 126.9 (C-2″), 109.3 (C-6′), 102.9 (C-2′), 102.5 (C-7′), 70.9 (C-2), 56.7 (C-8′), 20.3 (C-5′), 17.3 (C-3), 15.6 (C-4″).

High Performance Liquid Chromatography (HPLC). The HPLC apparatus (Jasco, Gross-Umstadt, Germany) consisted of a MD-2010 plus photodiode array detector and two PU 2087 pumps. Chromatographic separations were performed on 250 × 4.6 mm i.d. stainless-steel columns packed with Hyperclone 5 μm, RP-18 material for analytical scale (1.0 mL/min) and 250 × 21.2 mm i.d. stainless-steel columns packed with Microsorb, 5 μm, RP-18 material (Varian, Darmstadt, Germany) for preparative scale (18 mL/min).

LC−Time-of-Flight Mass Spectrometry (LC−TOF-MS). High resolution mass spectra of the compounds were measured on a Bruker Micro-TOF (Bruker Daltronics, Bremen, Germany) mass spectrometer and referenced to sodium formate.

LC−MS/MS. Electrospray ionization (ESI) mass and product ion spectra were acquired on an API 4000 QTRAP mass spectrometer (Applied Biosystems, Darmstadt, Germany) with direct flow infusion. The ion spray voltage was set at −4500 V in the negative mode and at 5500 V in the positive mode. The mass spectrometer was operated in the full-scan mode detecting positive or negative ions. The MS/MS parameters were set to induce fragmentation of the $[M - H]^-$ or $[M + H]^+$ molecular ions into specific product ions after collision with nitrogen as collision gas (4×10^{-5} Torr).

Nuclear Magnetic Resonance Spectroscopy (NMR). ^{1}H, ^{13}C and 2D-NMR data were acquired on a Bruker DPX-400 (Bruker BioSpin, Rheinstetten, Germany). $CDCl_3$ and pyridine-d_5 were used as solvents, and chemical shifts were referenced to the solvent signal. For structural elucidation and NMR signal assignment, COSY, HMQC, and HMBC experiments were carried out using the pulse sequences taken from the Bruker software library. Data processing was performed by using XWin-NMR software (version 3.5; Bruker, Rheinstetten, Germany) as well as Mestre-C (Mestrelab Research, A Coruña, Spain). 8-*O*-Angeloyl-tovarol (**13**) was measured on a JEOL-ECA 800 (Jeol, Tokyo, Japan), and data processing was performed by using the Delta NMR software (version 4.3.6; Jeol, Tokyo, Japan).

RESULTS AND DISCUSSION

Aimed at mapping the bitter taste compounds causing the bitter off-taste of carrots, a selection of fresh carrot samples was sensorially evaluated for the intensity of the bitter off-taste. The samples exhibiting the strongest bitterness were pooled, and then sequentially extracted with *n*-pentane, followed by ethyl

acetate to obtain the pentane soluble fraction A, the ethyl acetate soluble fraction B, as well as the nonsoluble residue (fraction C). After removing trace amounts of solvents, the individual fractions A, B, and C were taken up in water in their "natural" concentration ratios on a weight basis and evaluated for their bitter taste activity (**Table 1**). By far the highest bitter impact was found for fraction A judged with a score of 4.5, followed by fraction B exhibiting bitterness in a comparatively low intensity of 1.0. In contrast, the sensory panel did not detect any bitter taste in fraction C.

Sensory-Directed Deconstruction of Fraction A. In order to gain a more detailed insight into the bitter compounds present in fraction A, the pentane solubles were further fractionated by column chromatography on silica gel using mixtures of *n*-pentane and diethyl ether as mobile phase to give the five subfractions A-I to A-V. After solvent removal, the individual subfractions were taken up in water in their natural concentration ratios and were evaluated for their bitter impact. Fractions A-III, A-IV, and A-V showed rather high bitter intensities of 3.0, 4.5, and 4.0, whereas fractions A-I and A-II were either tasteless or exhibited just a very low bitterness (**Table 1**). As preliminary HPLC analysis revealed that fractions A-IV and A-V were substantially equivalent, both fractions were combined to give fraction A-IV/V.

Following this, the individual fractions A-III and A-IV/V, showing the highest sensory impacts, were separated by means of HPLC on RP-18 material into 14 fractions each (**Figure 2**). Monitoring the absorbance at 200 nm, the effluents of the individual HPLC analyses were collected, separated from solvent using vacuum, aliquots of the isolates were taken up in water, and their bitter intensity were evaluated sensorially (**Tables 2** and **3**). Among the HPLC fractions isolated from fraction A-IV/V, the subfractions A-IV/V/7 and A-IV/V/6 were rated with high scores of 5.0 and 4.5 for bitterness (**Table 2**), followed by fractions A-IV/V/4, A-IV/V/5, and A-IV/V/1 evaluated with bitter intensities of 4.0 and 3.5, respectively. Among the HPLC fractions obtained from fraction A-III, the subfractions A-III/1, A-III/8, and A-III/12 exhibited the highest bitter taste impact judged with a score of 5.0, followed by fractions A-III/4, A-III/5, and A-III/7 evaluated with a somewhat lower bitter taste impact (<3.5) (**Table 3**). All the other HPLC fractions isolated from A-III showed lower bitterness or were entirely tasteless.

Further investigation of the structure of key bitter compounds was made on fractions with high impact for sensory-perceived bitterness. After isolation and purification by means of rechromatography on RP18 material, the chemical structures of the individual bitter-tasting target compounds were determined on the basis of LC−MS/MS and 1D/2D-NMR spectroscopic experiments.

First, the identification experiments were focused on bitter tasting fractions isolated from fraction A-IV/V (**Table 2**). LC−MS analysis of the intensely bitter tasting fraction A-IV/V/1 revealed *m/z* 209 as the pseudomolecular ion $[M + H]^+$. Signal integration in the ^1H NMR spectrum and the number of resonance signals in the ^{13}C NMR spectrum revealed the presence of twelve hydrogen atoms and eleven carbon atoms, thus indicating an empirical formula of $C_{11}H_{12}O_4$. LC−TOF-MS analysis of that compound revealed a mass of *m/z* 231.0628 for the pseudomolecular ion $[M + Na]^+$, confirming a molecular formula of $C_{11}H_{12}O_4Na$. Comparison of chromatographic (RP-HPLC) and spectroscopic data (NMR, LC−MS, UV/vis) as well as cochromatography with the corresponding reference compound isolated recently (*4*) led to the unequivocal identification of 6-methoxymellein (**1**) (**Figure 1**) as the key bitter compound in fraction A-IV/V/1 (**Table 2**).

After isolation and purification of the bitter compound from fraction A-IV/V/7, comparison of chromatographic (RP-HPLC) and spectroscopic (LC−MS, UV/vis) with those of the reference compound, followed by cochromatography unequivocally led to the identification of falcarindiol (**2**) (**Figure 1**), recently reported as a bitter compound in carrots (*4*).

LC−MS analysis of the bitter compound isolated from fraction A-IV/V/6 revealed *m/z* 357 ($[M + Na]^+$) and *m/z* 352 ($[M + NH_4]^+$) as pseudomolecular ions. The ^1H and ^{13}C NMR spectra exhibited eleven signals in the proton dimension integrating for a total of 30 protons and 20 carbon atoms, where six were found to be quaternary carbons by means of a DEPT experiment. Taking all these data into account, an empirical formula of $C_{20}H_{30}O_4$ was suggested and, by comparison with the data reported in literature (*13*), the bitter tasting compound in fraction A-IV/V/6 could be unequivocally identified as vaginatin (**5**) (**Figure 3**). Although this sesquiterpene was isolated earlier from *Ferula communis* subsp. *communis* (*12*) and purple carrots (*13*), to the best of our knowledge vaginatin has not been previously reported as a bitter compound.

LC−TOF-MS analysis of the bitter compound isolated from fraction A-IV/V/5, judged with an intense bitter taste of 4.0 (**Table 2**), delivered an exact mass of 357.2036 Da, fitting well with the formula of $C_{20}H_{30}O_4Na$. 1D- and 2D-NMR spectroscopy confirmed the presence of 30 protons and 20 carbons in the target sensometabolite. Interestingly, comparison of these spectroscopic data with those obtained from vaginatin (**5**) indicated identical carbon skeleton. The only major differences in the set of spectroscopic data were observed in the angeloyl moiety of vaginatin. The proton H−C(3′) resonated in the ^1H NMR spectrum of the target compound at 6.74 ppm and differed from the chemical shift of 6.02 ppm as observed for vaginatin. The COSY spectrum indicated a coupling between H−C(3′) and the methyl groups H−C(4′) and H−C(5′) with a coupling constant of 7.1 and 1.5 Hz, respectively. As the heteronuclear coupling between carbon C(1′) observed at 166.1 ppm and H−C(4) indicated that the ester moiety in the target compound is bound at the same carbon position as found in vaginatin, the angelic acid moiety in vaginatin was proposed to be replaced by an isomeric tiglic acid moiety in the novel taste compound. On the basis of these considerations, the bitter compound in fraction A-IV/V/5 was identified as (*E*)-((1*R*,3a*R*,4*R*,8a*S*,*Z*)-8a-hydroxy-1-isopropyl-3a,6-dimethyl-3-oxo-1,2,3,3a,4,7,8,8a-octahydroazulen-4-yl) 2-methylbut-2-enoate (**6**) (**Figure 3**). To the best of our knowledge, this compound, coined isovaginatin, has not been previously reported in the literature.

LC−MS analysis of the sensory impact molecules isolated from fractions A-IV/V/4 and A-IV/V/3, imparting bitter taste intensity of 4.0 and 3.0 (**Table 2**), respectively, revealed a molecular mass of 406 Da each. 1D- and 2D-NMR experiments of both fractions revealed eleven proton signals integrating for 26 protons as well as 21 carbon atoms including eight quaternary carbons, thus indicating an empirical formula of $C_{21}H_{26}O_8$ for both taste molecules. Interestingly, both compounds showed differences in the coupling constant between the protons H−C(1) and H−C(2), e.g. the compound isolated from fraction A-IV/V/4 showed a coupling constant of 5.1 Hz, whereas a coupling constant of 7.1 Hz was measured for the tastant isolated from fraction A-IV/V/3. Careful assignment of all the spectroscopic data and comparison with data reported earlier (*14*) led to the identification of 2-epilaserine oxide (**7**) and laserine oxide (**8**) as key bitter molecules in fractions A-IV/V/4 and A-IV/V/3

(**Figure 3**). Previously these compounds have been isolated from purple carrots (*13*), but their bitter taste activity has not been elucidated.

Analysis of the bitter tasting key compounds in fractions A-IV/V/8 to A-IV/V/11 by means of LC−MS revealed a molecular mass of 418 Da for all four molecules. The ^{1}H NMR spectra of each compound exhibited 20 signals integrating for 38 protons each. ^{13}C NMR studies as well as heteronuclear chemical shift correlation experiments showed 25 carbon atoms, among which seven were quaternary carbons. Comparison of all the spectroscopic data of these taste compounds with data reported earlier in the literature (*15*) led to the identification of 6-*O*,8-*O*-ditigloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**9**) and 6-*O*-angeloyl-, 8-*O*-tigloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**10**) as the key bitter compounds in fraction IV/8 and A-IV/V/9, respectively (**Figure 3**). LC−TOF-MS analysis of the bitter compound isolated from fraction A-IV/V/10 revealed an exact mass of 441.2611 Da, thus confirming the formula of $C_{25}H_{38}O_5Na$ and indicating the same C_{15}-sesquiterpene skeletal structure esterified with two C_5-moieties as found for compounds **9** and **10**. The HMBC experiment revealed a coupling between proton H−C(6) resonating at 5.80 ppm and the carbon atom C(1′) observed at 167.1 ppm, which showed a coupling with the methyl protons H−C(5′) and H−C(4′) resonating at 1.73 and 1.71 ppm, respectively. The double quartet detected at 6.70 ppm was observed to exhibit heteronuclear couplings in the HMBC spectrum with the methyl groups at 1.73 and 1.71 ppm and could be assigned to the proton H−C(3′). Hence the ester moiety at H−C(6) could be assigned to tiglic acid. The same coupling pattern was found for proton H−C(8) resonating at 5.67 ppm, but the chemical shifts of the methyl groups H−C(5″) and H−C(4″), observed at 1.91 and 2.02 ppm, as well as of the proton H−C(3″) detected at 6.10 ppm were different. Based on this coupling pattern, a angelic acid ester moiety was identified as part of the molecule structure. Careful consideration of all the spectroscopic data enabled the identification of 6-*O*-tigloyl, 8-*O*-angeloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**11**) as the bitter compound in fraction A-IV/V/10 (**Figure 3**). Spectroscopic analysis of the compound isolated from fraction A-IV/V/11 revealed an exact mass of 441.2614 Da, thus indicating the same formula as found for compound **11**. Proton H−C(3′) resonating at 6.02 ppm exhibited couplings with the methyl groups H−C(4′) and H−C(5′) detected at 1.95 and 1.74 ppm (**Figure 4**). In addition, carbon atom C(1′) detected at 166.7 ppm showed HMBC couplings with both methyl groups as well as proton H−C(6). According to the data published in the literature (*15*), carbon atom C(6) was esterified with an angelic acid moiety, thus leading to the identification of the bitter compound in fraction A-IV/V/11 as the 6-*O*-,8-*O*-diangeloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**12**) (**Figure 3**). Although compounds **9** and **10** previously have been reported as a natural product of *Anisotome lyallii* (*15*), these bitter compounds have not yet been identified in carrots. In addition, compounds **11** and **12** have not been previously reported.

After identification of the key bitter compounds in fraction A-IV/V, the following experiments were focused on the structure determination of the bitter molecules in fraction A-III (**Table 3**). Comparison of chromatographic (RP-HPLC), spectroscopic data (LC−MS, UV/vis), cochromatography with the corresponding reference compound led to the unequivocal identification of 6-methoxymellein (**1**), falcarindiol-3-acetate (**4**), and falcarinol (**3**) as the key bitter compounds in fraction A-III/1,

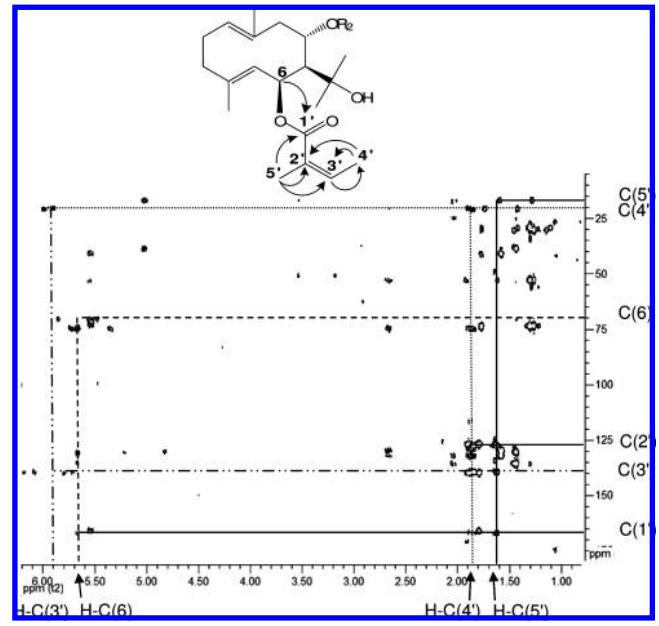

Figure 4. Section of HMBC spectrum (400 MHz, $CDCl_3$) of 6-, 8-*O*-diangeloyl-6*ss*,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**12**).

A-III/9, and A-III/13, respectively (**Table 3**), previously reported as bitter compounds in carrots (*4*).

LC−TOF-MS analysis of the intense bitter tasting compound isolated from fraction A-III/12 (**Table 3**) revealed *m*/*z* 343.2107 ($[M + Na]^+$) as the pseudomolecular ion correlating with a formula of $C_{20}H_{32}O_3Na$ with a calculated mass of 343.2244 Da. ^{1}H, ^{13}C, and DEPT-NMR spectroscopic experiments revealed 19 signals, which integrated for a total of 32 protons, and 20 carbon atoms including three quaternary carbons. In a heteronuclear multiple bond correlation (HMBC) experiment, the proton H−C(3′), resonating at 6.17 ppm and exhibiting a ^{1}J-coupling with carbon C(3′) at 140 ppm, was observed to show coupling with the carbon of two methyl groups resonating at 20.2 and 15.8 ppm, respectively. Carbon atom C(1′) resonating at 168 ppm showed ^{3}J-couplings with the methyl group H−C(5′) at 1.94 ppm as well as the proton H−C(8) observed at 5.24 ppm as expected for the coupling pattern of an angelic acid ester. In addition, a COSY experiment identified homonuclear coupling between proton H−C(8) and H−C(6) at 4.49 ppm as well as $H_{A/B}$-C(9) resonating at 2.09/2.63 ppm. Moreover, homonuclear couplings were detectable between H−C(6) and H−C(7), which showed homonuclear coupling with H−C(11) as part of a isopropyl moiety. Proton H−C(5) resonating at 5.29 ppm revealed a heteronuclear ^{1}J-coupling to C(5) at 133.3 ppm, a homonuclear ^{3}J-coupling to H−C(6), as well as a ^{4}J-coupling to the methyl group H−C(15) observed at 1.47 ppm. Quarternary carbon atom C(4) at 133.3 ppm and C(5) revealed $^{2,3}J$-couplings to H−C(6), heteronuclear $^{2,3}J$-couplings to H−C(15), $^{2,3}J$-couplings to $H_{A/B}$-C(3) and $^{3,4}J$-couplings to $H_{A/B}$-C(2). Protons $H_{A/B}$-C(3) at 2.20/2.11 ppm, H−C(15) and H−C(5) revealed couplings to C(3) at 38.4 ppm. Couplings between H_A-C(2) at 2.39 ppm, H_B-C(2) at 2.13 ppm and the olefinic proton H−C(1) at 5.06 ppm could be observed. H−C(1) revealed a homonuclear coupling with the methyl group H−C(14) at 1.69 ppm. C(10) at a chemical shift of 128.9 ppm and C(1) revealed a coupling to H−C(14), hence C(14) was bound to C(10). Both carbons also revealed couplings to H_A-C(9) and H_B-C(9). Careful consideration of all the NMR data demonstrated that the skeletal structure of the bitter target compound consisted of a ten-membered ring. Taking these data into account, the bitter compound in fraction A-III/12 could be identified as (*Z*)-

((1*S*,3*E*,7*E*,9*S*,10*S*)-9-hydroxy-10-isopropyl-3,7-dimethylcyclodeca-3,7-dienyl) 2-methylbut-2-enoate (**13**, **Figure 3**). Although this compound, known as 8-*O*-angeloyl-tovarol, was isolated earlier from *Thapsia villosa* (*16*), it has been neither described previously in carrots, nor has its bitter activity been reported.

LC−MS analysis of fraction A-III/6 and A-III/7, judged with a bitter taste intensity of 1.5 and 3.5 (**Table 3**), respectively, both revealed *m*/*z* 413 as the pseudomolecular ion $[M + Na]^+$, thus indicating a molecular weight of 390 Da. 1D- and 2D-NMR spectroscopic studies on both compounds revealed 26 protons and 21 carbons including eight quaternary carbon atoms. Both compounds showed differences in the coupling constant between H−C(1) and H−C(2), e.g. coupling constants of 7.1 and 4.1 Hz were measured for the compound isolated from fraction A-III/6 and A-III/7, respectively. As these two tastants showed rather similar coupling patterns in the 2D-NMR spectra as the compounds **7** and **8** but exhibited a by 16 Da decreased molecular mass, the taste compounds were expected to lack one oxygen atom. By comparison of all spectroscopic data with those reported in the literature (*17*), laserine (**14**) and 2-epilaserine (**15**) were identified as the key bitter compounds in fraction A-III/6 and A-III/7, respectively (**Figure 3**). Although both compounds have recently been isolated from purple carrots (*13*), their bitter taste activity was previously not known.

LC−MS and 1D/2D-NMR analysis of the tastant isolated form fraction A-III/3 (**Table 3**) revealed a molecular mass of 306 Da and the presence of 18 protons as well as 16 carbon atoms, of which six were identified as quaternary carbons by means of a DEPT-135 experiment. Based on these findings, an empiric formula of $C_{16}H_{18}O_6$ was proposed for the target molecule. Proton H−C(2) at 5.86 ppm revealed a ^1J-coupling to C(2) at 70.9 ppm, a coupling to an angelic acid moiety as in **14** and **15**. Further on H−C(2) showed homonuclear ^3J-coupling with methyl group H−C(3) at 1.49 ppm and ^2J-coupling to C(1) at 195 ppm. C(1) revealed heteronuclear ^3J-couplings to H−C(6′) at 7.09 ppm and H−C(2′) at 7.22 ppm, respectively. H−C(6′) and methoxy group H−C(8′) at 3.87 ppm exhibited heteronuclear $^{2,3}J$-coupling with C(5′). Proton H−C(7′) at 6.0 ppm, exhibiting an integration of two, revealed ^1J-coupling with C(7′) at 102.5 ppm, ^3J-couplings to C(3′) at 149 ppm and C(4′) at 139.9 ppm. H−C(7′) could be assigned to a methylendioxy function bound to the aromatic ring C(1′) - C(6′) at carbons C(3′) and C(4′). Careful interpretation of all LC−MS and 1D/2D-NMR data led to the identification of the bitter compound in fraction A-III/3 as (*Z*)-1-(4-methoxybenzo[*d*][1,3]dioxol-6-yl)-1-oxopropan-2-yl 2-methylbut-2-enoate (**16**) (**Figure 3**), the spectroscopic data of which was well in line with those reported previously (*18*). To the best of our knowledge, this compound, named α-angeloyloxy-latifolone, was previously not reported to exhibit bitter taste activity.

Although fractions A-III/4, A-III/5, and A-III/8 exhibited bitterness, the bitter compounds could not be identified, because the amounts isolated were too low for an unequivocal structure elucidation.

Bitter Recognition Threshold Concentrations. Prior to sensory analysis, the purity of the individual taste compounds was checked by ^{1}H NMR spectroscopy as well as HPLC-ELSD. To determine the human threshold concentrations for bitter taste, aqueous solutions of the compounds **1**−**16** were evaluated by means of a triangle test (**Table 4**). Depending on the chemical structure of these molecules, bitter taste thresholds were found to be between 8 and 200 μmol/L. Among these bitter compounds, the trihydroxygermacrane diene (**9**), 8-*O*-angeloyl-tovarol (**13**), and 2-epilaserine oxide (**7**) showed the lowest recognition thresholds below 15.0 μmol/L. The other compounds exhibited their bitter thresholds in the narrow range between 20 and 47 μmol/L with the exception of falcarinol (**3**) and falcarindiol-3-acetate (**4**), respectively, which showed threshold concentrations of 80 and 200 μmol/L confirming our previously published data (*4*) (**Table 4**).

Table 4. Human Taste Recognition Thresholds of Bitter Compounds Isolated from Carrots

compound (no.)[a]	threshold concn [μmol/kg][b]
falcarindiol-3-acetate (**4**)	200
falcarinol (**3**)	80
6-*O*-tigloyl-8-*O*-angeloyl- 6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**11**)	47
vaginatin (**5**)	45
falcarindiol (**2**)	40
laserine oxide (**8**)	37
laserine (**14**)	34
6,8-*O*-diangeloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**12**)	27
isovaginatin (**6**)	26
α-angeloyloxy-latifolone (**16**)	22
6-*O*-angeloyl-8-*O*-tigloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**10**)	21
6- methoxymellein (**1**)	20
2-epilaserine (**15**)	20
2-epilaserine oxide (**7**)	14
8-*O*-angeloyl-tovarol (**13**)	11
6,8-*O*-ditigloyl-6β,8α,11-trihydroxygermacra-1(10)*E*,4*E*-diene (**9**)	8

[a] Compound numbering refers to the structures given in **Figures 1** and **3**. [b] The bitter taste threshold was determined in aqueous solution by means of a triangle test.

Using a sensory-directed fractionation approach, a total of 16 bitter sensometabolites were identified in carrots, among which the bitter taste activity of 12 compounds was previously not reported. Studies on the sensory contribution of these sensometabolites to the bitter taste of various types of carrots are currently in progress and will be published separately.

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Received for review July 28, 2008. Revised manuscript received September 12, 2008. Accepted September 12, 2008. We gratefully acknowledge the German Research Foundation (DFG) for partly funding this project in the framework of the International Research Training Group Münster/Germany-Nagoya/Japan (GRK 1143).

JF8023358