JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

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pubs.acs.org/JAFC

Key Phytochemicals Contributing to the Bitter Off-Taste of Oat (*Avena sativa* L.)

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ABSTRACT: Sensory-directed fractionation of extracts prepared from oat flour (*Avena sativa* L.) followed by LC-TOF-MS, LC-MS/MS, and 1D/2D-NMR experiments revealed avenanthramides and saponins as the key phytochemicals contributing to the typical astringent and bitter off-taste of oat. Besides avenacosides A and B, two previously unreported bitter-tasting bidesmosidic saponins were identified, namely, 3-(*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl(1→4)]-β-D-glucopyranosid)-26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol, and 3-(*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-β-D-glucopyranosid)-26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol. Depending on the chemical structure of the saponins and avenanthramides, sensory studies revealed human orosensory recognition thresholds of these phytochemicals to range between 3 and 170 μmol/L.

KEYWORDS: *oat, Avena sativa, saponin, avenanthramide, taste, taste dilution analysis, bitter, astringent, avenacoside*

INTRODUCTION

Within the past years, oats (*Avena sativa* L.) have become fairly popular among cereal crops and are consumed as a key ingredient of breakfast cereals such as porridge and muesli, cookies, breads, and oat drinks, respectively. Besides nutritionally favorable amounts of proteins, soluble fiber, unsaturated fatty acids, vitamins, minerals, and polyphenols, oats have been reported to show health-promoting attributes in blood cholesterol and coronary heart diseases.[1,2] Moreover, oats are appreciated for their characteristic flavor, which is described with a nutty, sweet, and cereal-like aroma.[3] This aroma is paired with a slight sweet, bitter, and astringent after-taste that is considered an off-taste if perceived as being too intense.

Mainly lipid-derived compounds are assumed to contribute to the typical bitterness of oat and oat fibers, among them 9-hydroxy-*trans*,*cis*-10,12-octadecadienoic-1′-monoglyceride, 13-hydroxy-*cis*,*trans*-9,11-octadecadienoic-1′-monoglyceride, 9-hydroxy-*trans*,*cis*-10,12-octadecadienoic acid, 13-hydroxy-*cis*,*trans*-9,11-octadecadienoic acid, 9,12,13-trihydroxy-*trans*-10-octadecenoic acid, and 9,10,13-trihydroxy-*trans*-11-octadecenoic acid.[4−8] Moreover, glycosidic phytochemicals, such as avenacosides A and B, have been isolated from oat and hypothesized to contribute to the characteristic bitter taste.[9,10] Although the content of phenolic compounds seems to show some correlation of avenanthramides with perceived bitterness, freshness, and rancidity of oats,[11] the knowledge of individual key taste molecules imparting the bitter taste of oats is rather fragmentary. To selectively sort out such key taste molecules from the bulk of sensorially inactive components in foods, the so-called taste dilution analysis (TDA)[12] has been developed and successfully applied in recent years to elucidate the most important tastants such as bitter compounds in asparagus,[13−15] avocado,[16] and hops,[17,18] pungent and tingling phytochemicals in black pepper,[19] and astringent molecules in roasted cocoa nibs[20] and red wine.[21]

Therefore, the objective of the present study was to locate the key molecules responsible for the typical astringent and bitter taste of oats by application of the taste dilution technique, to determine the chemical structure of the most taste-active phytochemicals by means of LC-MS/MS, UPLC-TOF-MS and 1D/2D-NMR experiments, and to evaluate their sensory activity on the basis of their human recognition thresholds.

MATERIALS AND METHODS

Chemicals and Materials. The following compounds were obtained commercially: formic acid, hydrogen chloride, sodium chloride, sodium bicarbonate, caffeine, L-lactic acid (Merck, Darmstadt, Germany); L-glutamic acid monohydrate, anthranilic acid, 5-hydroxyanthranilic acid, Meldrum's acid, *p*-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, syringaldehyde, vanillin, *p*-anisaldehyde, pyridine, toluene (Sigma-Aldrich, Steinheim, Germany); sucrose (Carl Roth, Karlsruhe, Germany); sulfuric acid, hydrochloric acid, β-alanine, and sodium bicarbonate (Merck). Deuterated solvents were supplied by Euriso-Top (St. Aubin, France). Solvents for HPLC applications were of HPLC grade (Merck), and solvents for LC-MS uses were of LC-MS grade (J. T. Baker, Deventer, The Netherlands), whereas solvents used for extraction (Merck) were distilled prior to use. Water for HPLC separation was purified by means of a Milli-Q water advantage A 10 water system (Millipore, Molsheim, France). Bottled water (Evian, low mineralization = 405 mg/L) was adjusted to pH 6.4 with aqueous formic acid prior to gustatory analysis. Ethanol (absolute, Merck) was utilized for sensory analysis. Ground oat flour from heat-treated oat grains was used and stored at −20 °C until use.

Analytical Sensory Analyses. *Training of the Sensory Panel.* According to the literature[22] and to familiarize the panelists (six females, six males; 22−35 years in age) with the taste language, assessors were trained on different qualities of oral sensation. The

Received: November 7, 2016
Revised: December 2, 2016
Accepted: December 6, 2016
Published: December 6, 2016

DOI: 10.1021/acs.jafc.6b04995
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

panelists, who had no history of known taste disorders and had given informed consent to participate in the following sensory studies, took part in weekly sessions for at least two years. Aqueous solutions (2 mL; pH 6.4) of the following standard taste components were used for training: sucrose (50 mM) for sweet taste, L-lactic acid (20 mM) for sour taste, NaCl (20 mM) for salty taste, caffeine (1 mM) for bitter taste, and sodium glutamate (3 mM) for umami taste. For the astringent oral sensation, tannic acid (0.05%) was used to train the panelists using the so-called half-tongue test.[21,22] All sensory analyses were performed in a sensory panel room at 22−25 °C in three different sessions using nose clips to avoid cross-model interactions with odorants.

Pretreatment of Food Fractions and Taste Compounds for Sensory Analysis. Prior to sensory analysis, fractions or compounds isolated from oat flour were suspended in water and freeze-dried twice, after removal of the volatiles in high vacuum (<5 mPa). ^{1}H NMR and ion chromatography revealed that food fractions treated by that procedure are essentially free of solvents and buffer compounds.

Taste Profile Analysis. Oat flour (15 g) was suspended in water (50 mL, pH 6.4) containing 3% ethanol and, after filtration (folded paper filter, 125 mm; Machery Nagel, Düren, Germany), the aqueous supernatant was presented to the trained sensory panel to evaluate the taste sensations sweet, sour, umami, salty, bitter, and astringent on a linear intensity scale from 0 (not detectable) to 5 (strongly detectable). In the same way, aliquots of oat fractions I−IV, taken up in "natural" concentrations in bottled water (30 mL; pH 6.4, 3% ethanol), were offered to rate the taste intensities.

Taste Dilution Analysis (TDA). Aliquots of the HPLC fractions were taken up in "natural" concentrations in bottled water (6.0 mL; pH 6.4, 1% ethanol) and, then, sequentially diluted 1:1 (v/v) with bottled water (pH 6.4, 1% ethanol). The series of dilutions were presented randomly to the trained panel in order of increasing concentration. Panelists were asked to evaluate the dilution step at which a difference between the sample and a control (water, pH 6.4, 1% ethanol) could just be detected by means of the half-tongue test.[21−23] The so-called taste dilution (TD) factors were calculated by the geometric mean of all individual threshold concentrations in separate sessions and did not differ by more than plus/minus one dilution step.[24,25]

Solvent Extraction of Oat Flour. A mixture of oat flour (300 g) and freshly distilled *n*-hexane (1 L) was vigorously stirred for 3 h at room temperature, followed by centrifugation for 25 min at 4 °C and cold filtration. The filtrates were collected, and the procedure was repeated three times to give the hexane extractables (fraction I). The residual oat flour was extracted three times (1 L) for 8 h with a methanol/water mixture (70:30, v/v) adjusted to pH 4.0 with aqueous formic acid. After filtration, the combined filtrates were removed from solvent by vacuum evaporation at 40 °C, followed by lyophilization to give the methanol/water extractables (fraction II). The residue was extracted with methanol (3 × 1 L) and, after filtration, the combined filtrates were separated from solvent in vacuum, followed by lyophilization to give the methanol extractables (fraction III). After lyophilization of the remaining oat residue, insoluble fraction IV was obtained. The individual fractions I−IV were freeze-dried twice to remove trace amounts of solvents. Their yields were determined by weight, and their taste profiles were evaluated in aqueous solutions as given in Table 1.

Solid Phase Separation of Oat Fraction II. An aliquot (900 mg) of freeze-dried oat fraction II was suspended in 0.1% aqueous formic acid (10 mL) and applied onto a Strata C18-E cartridge (10 g/60 mL, Phenomenex, Aschaffenburg, Germany), preconditioned with methanol (60 mL), followed by methanol/water (50:50, v/v, 60 mL), and 0.1% aqueous formic acid (60 mL). Using a vacuum extraction box (J. T. Baker, Phillipsburg, NJ, USA), the cartridge was flushed stepwise with volumes (30 mL) of 0.1% aqueous formic acid to give fraction II-A (yield 67.6%), methanol/aqueous formic acid (40:60, v/v) to give fraction II-B (yield 7.8%), methanol/aqueous formic acid (60:40, v/v) to give fraction II-C (yield 3.0%), methanol/aqueous formic acid (80:20, v/v) to give fraction II-D (yield 3.0%), and methanol to give fraction II-E (yield 3.9%). Fractions were collected separately, freed from solvent under vacuum, taken up in water, and lyophilized twice for sensory analysis. Yields were determined gravimetrically and extracts kept at −20 °C until used for sensory and chemical analysis, respectively.

Table 1. Yields and Sensory Evaluation of Fractions Isolated from Oat Flour

		fraction[a]			
taste intensity[b] of	oat flour	I	II	III	IV
bitterness	2.0	0.8	2.6	1.2	<0.1
astringency	1.1	1.0	1.2	0.7	<0.1
sweetness	0.5	0.6	0.5	0.5	<0.1
sourness	0.3	0.3	0.8	0.4	<0.1
saltiness	0.1	0.2	n.d.	0.2	<0.1
umami taste	0.1	0.1	0.4	0.3	<0.1

[a]Individual fractions obtained by solvent extraction of oat flour with *n*-hexane (fraction I, yield 8.1 g/100 g), methanol/water (fraction II, yield 2.5 g/100 g), and methanol (fraction III, yield 0.7 g/100 g) to give the nonsoluble residue (fraction IV, yield 84.2 g/100 g). [b]The taste intensity of aqueous solutions of the "natural" concentrations of individual fractions in bottled water (pH 6.4, 3% ethanol) was rated on a scale from 0 (not detectable) to 5 (strongly detectable).

Identification of Key Taste Compounds in Subfraction II-D. Oat subfraction II-D was dissolved in acetonitrile/water (20:80, v/v; 24 mg/600 μL) and, after membrane filtration, was injected onto a 250 × 21.0 mm, 5 μm, Nucleodur C18 Pyramid column (Machery-Nagel, Düren, Germany) equipped with a guard column of the same type and operated with a flow rate of 19 mL/min. Using 0.1% aqueous formic acid as solvent A and acetonitrile as solvent B, chromatography was performed with the following gradient: 0 min, 23% B; 6 min, 30% B; 16 min, 36% B; 20 min, 65% B; 22 min, 100% B; 24 min, 100% B; 26 min, 23% B; 30 min, 23% B. The effluent was separated into 16 subfractions (II-D-1−II-D-16), which were collected individually in multiple HPLC runs. Fractions were combined, removed from solvent under vacuum evaporation at 40 °C, and freeze-dried twice. The TDA was performed by dissolving fractions in water containing 1% ethanol in their "natural" concentration ratios. Bitter-tasting fractions (II-D-4, II-D-5, II-D-8, II-D-9) were further separated by means of preparative RP-HPLC using a 250 × 21.0 mm, 5 μm, Nucleodur C18 Pyramid column (Machery-Nagel) equipped with a guard column of the same type. Using 0.1% aqueous formic acid as solvent A and acetonitrile as solvent B, chromatography was performed running an isocratic gradient of 19% (II-D-4, II-D-5) and 33% solvent B (II-D-8, II-D-9), respectively, for 12 min with a flow rate of 19 mL/min. Fractions were combined and separated from solvent under vacuum. After lyophilization, the structures of the key bitter compounds **1** (II-D-4), **2** (II-D-5), **3** (II-D-8), and **4** (II-D-9) were identified as 3-(*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl(1→4)]-β-D-glucopyranoside)-26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol (**1**), 3-(*O*-α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl(1→4)]-β-D-glucopyranoside)-26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol (**2**), and avenacosides B (**3**) and A (**4**) by means of LC-MS/MS, UPLC-TOF-MS, and 1D/2D NMR experiments.

3-(O-α-L-Rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3-β-D-glucopyranosyl(1→4)]-β-D-glucopyranoside)-26-O-β-D-glucopyranosyl-(25R)-furost-5-ene-3β,22,26-triol, 1 (Figure 1): LC-MS (ESI$^-$) *m/z* 1225.5 [M − H]$^-$, 1063.5 [M − Glc − H]$^-$, 901.4 [M − 2Glc − H]$^-$, 755.4 [M − 2Glc − Rha − H]$^-$, 531.2, 441.1; LC-MS (ESI$^+$) *m/z* 1249.5 [M + Na]$^+$, 1209.5 [M − 18 + H]$^+$, 1047.5 [M − Glc − 18 + H]$^+$, 901.4 [M − Glc − Rha − 18 + H]$^+$, 739.4 [M − 2Glc − Rha − 18 + H]$^+$, 577.3 [M − 3Glc − Rha − 18 + H]$^+$, 415.3 [M − 4Glc − Rha − 18 + H]$^+$; MS/MS (DP = −225 V) *m/z* (%) 1225.6 (100), 1063.6 (3), 901.4 (11), 755.4 (9), 575.6 (2), 289.0 (3); LC-TOF-MS *m/z* 1225.5867 ([M − H]$^-$, measured), *m/z* 1225.5853 calculated for [$C_{57}H_{94}O_{28}$ − H]$^-$. ^{1}H and ^{13}C NMR data are given in Tables 2 and 3.

DOI: 10.1021/acs.jafc.6b04995
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

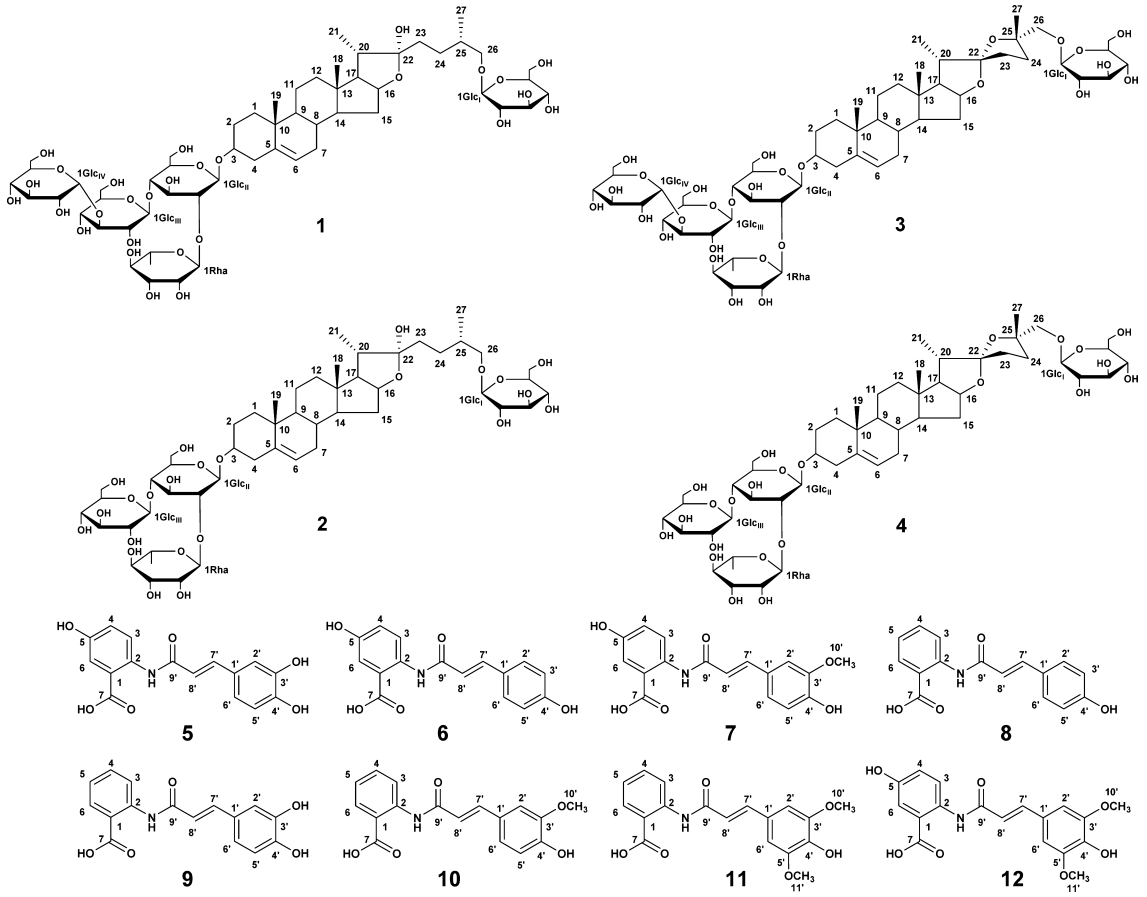

Figure 1. Chemical structures of 3-(*O*-*α*-L-rhamnopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→3)-*β*-D-glucopyranosyl(1→4)]-*β*-D-glucopyranosid)-26-*O*-*β*-D-glucopyranosyl-(25*R*)-furost-5-ene-3*β*,22,26-triol (**1**), 3-(*O*-*α*-L-rhamnopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→4)]-*β*-D-glucopyranosid)-26-*O*-*β*-D-glucopyranosyl-(25*R*)-furost-5-ene-3*β*,22,26-triol (**2**), nuatigenin-3-*O*-(*α*-L-rhamnopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl-(1→4)]-*β*-D-glucopyranoside)-26-*O*-*β*-D-glucopyranoside (avenacoside B, **3**), nuatigenin-3-*O*-(*α*-L-rhamnopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→4)]-*β*-D-glucopyranoside)-26-*O*-*β*-D-glucopyranoside (avenacoside A, **4**), avenanthramide 2c (**5**), avenanthramide 2p (**6**), avenanthramide 2f (**7**), avenanthramide 2f (**7**), avenanthramide 1p (**8**), avenanthramide 1c (**9**), avenanthramide 1f (**10**), avenanthramide 1s (**11**), and avenanthramide 2s (**12**).

*3-(O-α-L-Rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-β-D-glucopyranoside)-26-O-β-D-glucopyranosyl-(25R)-furost-5-ene-3β,22,26-triol, **2** (Figure 1).* LC-MS (ESI$^-$) *m*/*z* 1063.5 [M − H]$^-$, 901.4 [M − Glc − H]$^-$, 755.1 [M − Glc − Rha − H]$^-$, 593.3 [M − 2Glc − Rha − H]$^-$, 298.0; LC-MS (ESI$^+$) *m*/*z* 1087.5 [M + Na]$^+$, 1047.5 [M − 18 + H]$^+$, 885.4 [M − Glc − 18 + H]$^+$, 739.4 [M − Glc − Rha − 18 + H]$^+$, 577.3 [M − 2Glc − Rha − 18 + H]$^+$, 415.3 [M − 3Glc − Rha − 18+H]$^+$; MS/MS (DP = −185 V) *m*/*z* (%) 1064.2 (100), 902.2 (48), 755.8 (2), 575.2 (5), 431.2 (10), 289.6 (13); LC-TOF-MS *m*/*z* 1063.5334 ([M − H]$^-$, measured), *m*/*z* 1063.5325 calculated for [$C_{51}H_{84}O_{23}$ − H]$^-$. ^{1}H and ^{13}C NMR data are given in Tables 2 and 3.

*Nuatigenin-3-O-(α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→3)-β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside)-26-O-β-D-glucopyranoside (avenacoside B), **3** (Figure 1):* LC-MS (ESI$^+$) *m*/*z* 1063.5 [M − Glc + H]$^+$, 901.4 [M − 2Glc + H]$^+$, 739.4 [M − 3Glc + H]$^+$, 593.3 [M − 3Glc − Rha + H]$^+$, 431.3 [M − 4Glc − Rha + H]$^+$, 413.3, 271.2; MS/MS (DP = −200 V) *m*/*z* (%) 1223.6 (100), 1061.4 (9), 899.6 (12), 753.4 (2), 591.0 (1); LC-TOF-MS *m*/*z* 1223.5695 ([M − H]$^-$, measured), *m*/*z* 1223.5697 calculated for [$C_{57}H_{92}O_{28}$ − H]$^-$. ^{1}H and ^{13}C NMR data are given in Table 4 and 5.

*Nuatigenin-3-O-(α-L-rhamnopyranosyl-(1→2)-[β-D-glucopyranosyl-(1→4)]-β-D-glucopyranoside)-26-O-β-D-glucopyranoside or (avenacoside A), **4** (Figure 1):* LC-MS (ESI$^+$) *m*/*z* 1085.5 [M + Na + H]$^+$, 1063.5 [M + H]$^+$, 901.4 [M − Glc + H]$^+$, 739.4 [M − 2Glc + H]$^+$, 593.3 [M − 2Glc − Rha + H]$^+$, 431.3 [M − 3Glc − Rha + H]$^+$, 413.3, 271.2; MS/MS (DP = +85 V) *m*/*z* (%) 1064.4 (48), 902.4 (21) 756.2 (8), 594.4 (28), 431.4 (77) 413.4 (30); LC-TOF-MS *m*/*z* 1061.5169 ([M − H]$^-$, measured), *m*/*z* 1061.5168 calculated for [$C_{51}H_{82}O_{23}$ − H]$^-$. ^{1}H and ^{13}C NMR data are given in Tables 4 and 5.

Identification of Key Taste Compounds in Fraction II-C. Fraction II-C was dissolved in acetonitrile/water (20:80, v/v; 3 mg/80 μL) and, after membrane filtration, was injected onto a 250 × 10.0 mm, 4 μm, Synergi Hydro RP column (Phenomenex) equipped with a guard column of the same type operated with a flow rate of 6.5 mL/min. Using 0.1% aqueous formic acid as solvent A and acetonitrile as solvent B, chromatography was performed with the following gradient: 0 min, 20% B; 1 min, 22% B; 16 min, 30% B; 19 min, 40% B; 21 min, 100% B; 23 min, 100% B; 25 min, 20% B; 27 min, 20% B. The effluent was separated into 17 subfractions, namely, II-C-1−II-C-17, which were collected individually in several runs. The solvent was removed under vacuum evaporation at 40 °C. After freeze-drying twice, the fractions were used for sensory experiments, and fractions 5 (II-C-11), 6 (II-C-15), and 7 (II-C-16) were identified to contain avenanthramides 2c (**5**), 2p (**6**), and 2f (**7**) by means of UV−vis, LC-MS/MS, UPLC-TOF-MS, and 1D/2D NMR experiments, respectively.

*N-(3′,4′-Dihydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (avenanthramide 2c), **5** (Figure 1):* UV−vis (ACN) λ_{max} = 216 nm, 232 nm, 316 nm; LC-TOF-MS *m*/*z* 314.0669 ([M − H]$^-$, measured), *m*/*z* 314.0665 calculated for [$C_{16}H_{13}NO_6$ − H]$^-$; ^{1}H NMR (500 MHz, MeOD-d_4) *δ* 6.46 [d, 1H, *J* = 15.6 Hz, H−C(8′)], 6.79 [d, 1H, *J* = 8.3 Hz, H−C(5′)], 6.97 [dd, 1H, *J* = 2.1, 8.3 Hz, H−C(6′)], 7.00 [dd, 1H, *J* = 2.9, 9.0 Hz, H−C(4)], 7.07 [d, 1H, *J* = 2.0 Hz, H−C(2′)], 7.50 [d, 1H, *J* = 15.7 Hz, H−C(7′)], 7.51 [d, 1H, *J* = 2.9 Hz, H−C(6)], 8.44 [d, 1H, *J* = 9.0 Hz, H−C(3)]; ^{13}C NMR [125 MHz,

DOI: 10.1021/acs.jafc.6b04995
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Table 2. ^{1}H NMR (500 MHz, Pyridine-d_6) and ^{13}C NMR Signals (125 MHz, Pyridine-d_6) of the Aglycone Moiety of Saponins 1 and 2

	1[a,b]				2[a,b]			
position	δ_C	HSQC	δ_H	M [J, Hz]	δ_C	HSQC	δ_H	M [J, Hz]
1	37.8	[CH_2]	0.94−1.04	m	37.8	[CH_2]	0.94−1.04	m
			1.70−1.79	m			1.70−1.79	m
2	30.4	[CH_2]	1.85−1.90	m	30.5	[CH_2]	1.85−1.91	m
			2.06−2.12	m			2.08−2.14	m
3	78.4	[CH]	3.83−3.93	m	78.4	[CH]	3.83−3.93	m
4	39.2	[CH_2]	2.68−2.81		39.2	[CH_2]	2.68−2.81	
			2.68−2.81				2.68−2.81	
5	141.1	[C]	–			[C]	–	
6	122.1	[CH]	5.27−5.32	d	122.1	[CH]	5.27−5.32	d
7	32.7	[CH_2]	1.39−1.52	m	32.7	[CH_2]	1.39−1.52	m
			1.82−1.93				1.82−1.93	
8	32.0	[CH]	1.52−1.63	m	32.0	[CH]	1.52−1.63	m
9	50.7	[CH]	0.87−0.95	m	50.7	[CH]	0.87−0.95	m
10	37.4	[C]	–		37.4	[C]	–	
11	21.3	[CH_2]	1.40−1.52	m	21.3	[CH_2]	1.38−1.52	m
			1.40−1.52				1.38−1.52	
12	40.2	[CH_2]	1.07−1.19	m	40.2	[CH_2]	1.08−1.17	m
			1.72−1.80	o			1.72−1.79	o
13	41.0	[C]	–		41.0	[C]	–	
14	56.9	[CH]	1.04−1.13	m	56.9	[CH]	1.00−1.11	m
15	32.8	[CH_2]	1.42−1.52	m	32.8	[CH_2]	1.41−1.52	m
			1.99−2.10	o			1.96−2.07	o
16	81.4	[CH]	4.92−5.00	–	81.4	[CH]	4.92−5.00	–
17	64.1	[CH]	1.91−1.99	t	64.1	[CH]	1.91−1.99	t
18	16.7	[CH_3]	0.88−0.93	s	16.7	[CH_3]	0.87−0.94	s
19	19.0	[CH_3]	1.02−1.11	s	19.0	[CH_3]	1.02−1.11	s
20	41.1	[CH]	2.22−2.29	m	41.1	[CH]	2.20−2.27	m
21	16.7	[CH_3]	1.28−1.42	d	16.7	[CH_3]	1.29−1.40	d
22	111.0	[C]	–		111.0	[C]	–	
23	37.4	[CH_2]	1.70−1.78	o	37.4	[CH_2]	1.70−1.78	o
			2.00−2.09	o			2.00−2.09	o
24	28.6	[CH_2]	1.68−1.76	m	28.6	[CH_2]	1.67−1.73	m
			2.04−2.11	o			2.03−2.08	o
25	34.6	[CH]	1.89−1.99	m	34.6	[CH]	1.89−1.97	m
26	75.5	[CH_2]	3.58−3.66	dd	75.5	[CH_2]	3.58−3.66	dd
			3.92−3.98	dd			3.92−3.98	dd
27	17.7	[CH_3]	0.98−1.02	d [J = 6.6]	17.7	[CH_3]	0.98−1.02	d [J = 6.6]

[a]Arbitrary numbering according to structures **1** and **2** in Figure 1. [b]Assignments were based on HSQC, HMBC, J-res, and COSY experiments. d, doublet; m, multiplet signals; o, overlapped with other signals; s, singlet; t, triplet.

MeOD-d_4) δ 115.1 [C(2′)], 116.5 [C(5′)], 118.2 [C(6)], 119.5 [C(8′)], 120.4 [C(1)], 121.6 [C(4)], 122.5 [C(6′)], 123.3 [C(3)], 128.1 [C(1′)], 134.7 [C(2)], 143.3 [C(7′)], 146.8 [C(3′)], 149.1 [C(4′)], 154.1 [C(5)], 166.8 [C(9′)], 171.7 [C(7)].

N-(4′-Hydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (avenanthramide 2p), ***6*** *(Figure 1):* UV−vis (ACN) λ_{max} = 216 nm, 320 nm; LC-TOF-MS m/z 298.0716 ([M − H]$^-$, measured), m/z 298.0715 calculated for [$C_{16}H_{13}NO_5$ − H]$^-$; ^{1}H NMR (500 MHz, MeOD-d_4) δ 6.53 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.82 [d, 2H, J = 8.6 Hz, H−C(3′/5′)], 6.98 [dd, 1H, J = 9.0, 3.0 Hz, H−C(4)], 7.48 [d, 2H, J = 8.6 Hz, H−C(2′/6′)], 7.51 [d, 1H, J = 3.0 Hz, H−C(6)], 7.56 [d, 1H, J = 15.6 Hz, H−C(7′)], 8.45 [d, 1H, J = 9.0 Hz, H−C(3)]; ^{13}C NMR (125 MHz, MeOD-d_4) δ 116.8 [C(3′/5′)], 118.3 [C(6)], 119.6 [C(8′)], 120.5 [C(1)], 121.3 [C(4)], 123.2 [C(3)], 127.6 [C(1′)], 130.8 [C(2′/6′)], 134.8 [C(2)], 142.8 [C(7′)], 154.2 [C(5)], 160.9 [C(4′)], 166.5 [C(9′)], 171.1 [C(7)].

N-(4′-Hydroxy-3′-methoxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (avenanthramide 2f), ***7*** *(Figure 1):* UV−vis (ACN) λ_{max} = 216 nm, 332 nm; LC-TOF-MS m/z 328.0851 ([M − H]$^-$, measured), m/z 328.0821 calculated for [$C_{17}H_{15}NO_6$ − H]$^-$; ^{1}H NMR (400 MHz, MeOD-d_4) δ 3.92 [s, 3H, H−C(10′)], 6.56 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.82 [d, 1H, J = 8.2 Hz, H−C(5′)], 6.99 [dd, 1H, J = 3.0, 9.0 Hz, H−C(4)], 7.09 [dd, 1H, J = 2.0, 8.1 Hz, H−C(6′)], 7.22 [d, 1H, J = 1.9 Hz, H−C(2′)], 7.51 [d, 1H, J = 3.0 Hz, H−C(6)], 7.56 [d, 1H, J = 15.6 Hz, H−C(7′)], 8.45 [d, 1H, J = 9.0 Hz, H−C(3)]; ^{13}C NMR (101 MHz, MeOD-d_4) δ 56.5 [C(10′)], 111.6 [C(2′)], 116.5 [C(5′)], 118.2 [C(6)], 119.3 [C(8′)], 119.6 [C(1)], 122.0 [C(4)], 123.5 [C(3)], 123.8 [C(6′)], 128.1 [C(1′)], 134.8 [C(2′)], 143.4 [C(7′)], 149.4 [C(3′)], 150.2 [C(4′)], 154.2 [C(5)], 166.8 [C(9′)], 171.2 [C(7)].

Thin Layer Chromatography (TLC). According to the literature,[26] TLC analysis was performed using a silica gel TLC plate 60 coated with fluorescent indicator F_{254} (Merck) and chloroform/methanol/water (8:4:1; v/v/v) as the mobile phase. After chromatographic separation, the TLC plate was sprayed with reagent solution comprising a mixture (85:10:5:0.8; v/v/v/v) of methanol, acetic acid, sulfuric acid, and p-anisaldehyde and heated in an oven for 2 min at 110 °C.

Acidic Hydrolysis of Compounds 1−4 and Carbohydrate Analysis. Trifluoroacetic acid (4 mL) was added to a mixture of the

DOI: 10.1021/acs.jafc.6b04995
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 3. ^{1}H NMR (500 MHz, Pyridine-d_6) and ^{13}C NMR Signals (125 MHz, Pyridine-d_6) of the Sugar Moieties of Saponins 1 and 2

			1[a,b]			2[a,b]		
position	δ_C	HSQC	δ_H	M [J, Hz]	δ_C	HSQC	δ_H	M [J, Hz]
β-D-GlcI (at C-26)								
1′	105.2	[CH]	4.80−4.85	d [J = 7.7]	105.2	[CH]	4.80−4.85	d [J = 7.7]
2′	75.3	[CH]	4.02−4.10	m	75.3	[CH]	4.02−4.10	o
3′	78.9	[CH]	3.93−3.98	o	78.9	[CH]	3.93−4.00	o
4′	71.9	[CH]	4.19−4.28	m	72.0	[CH]	4.18−4.28	m
5′	78.6	[CH]	4.19−4.28	m	78.6	[CH]	4.18−4.28	m
6′	62.8	[CH_2]	4.29−4.35	o	62.8	[CH_2]	4.30−4.37	o
			4.53−4.59	o			4.50−4.56	o
β-D-GlcII (at C-3)								
1″	100.3	[CH]	4.92−4.98	d [J = 7.2]	100.3	[CH]	4.92−4.98	d [J = 7.0]
2″	77.9	[CH]	4.20−4.27	m	77.9	[CH]	4.19−4.27	m
3″	76.5	[CH]	3.82−3.88	m	76.5	[CH]	3.82−3.88	m
4″	81.8	[CH]	4.20−4.26	m	82.3	[CH]	4.19−4.26	m
5″	77.6	[CH]	4.19−4.26	m	78.5	[CH]	4.19−4.27	m
6″	61.8	[CH_2]	4.43−4.50	m	62.2	[CH_2]	4.43−4.50	m
			4.50−4.55	m			4.50−4.55	m
β-D-GlcIII (at C-4GlcII)								
1‴	104.8	[CH]	5.08−5.13	d [J = 7.8]	105.5	[CH]	5.10−5.18	d [J = 7.8]
2‴	74.1	[CH]	4.04−4.09	m	75.3	[CH]	4.01−4.10	m
3‴	88.6	[CH]	4.16−4.21	m	78.0[a]	[CH]	4.19−4.26	m
4‴	69.6	[CH]	4.14−4.20	m	71.5	[CH]	4.22−4.30	m
5‴	78.3	[CH]	3.86−3.93	m	78.8	[CH]	3.91−4.01	m
6‴	62.4	[CH_2]	4.29−4.36	m	62.4	[CH_2]	4.29−4.36	m
			4.42−4.49	m			4.42−4.49	m
α-L-Rha (at C-2GlcII)								
1⁗	102.1	[CH]	6.22−6.27	bs [J = 2.2]	102.1	[CH]	6.22−6.27	bs [J = 2.3]
2⁗	72.7	[CH]	4.72−4.78	dd	72.7	[CH]	4.72−4.78	dd
3⁗	73.1	[CH]	4.55−4.63	dd	73.1	[CH]	4.55−4.63	dd
4⁗	74.4	[CH]	4.31−4.38	t.o	74.4	[CH]	4.31−4.38	t.o
5⁗	69.8	[CH]	4.90−4.98	o	69.8	[CH]	4.90−4.98	o
6⁗	18.9	[CH_3]	1.72−1.80	d	18.9	[CH_3]	1.72−1.80	d
β-D-GlcIV (at C-3GlcIII)								
1⁗′	106.2	[CH]	5.27−5.32	d [J = 8.0]				
2⁗′	75.5	[CH]	4.05−4.11	o				
3⁗′	78.4	[CH]	4.20−4.28	o				
4⁗′	72.0	[CH]	4.17−4.27	o				
5⁗′	78.9	[CH]	4.21−4.29	o				
6⁗′	62.8	[CH_2]	4.29−4.35	o				
			4.53−4.59	o				

[a]Arbitrary numbering according to structures **1** and **2** in Figure 1. [b]Assignments were based on HSQC, HMBC, *J*-res, and COSY experiments. d, doublet; m, multiplet signals; o, overlapped with other signals; bs, broad singlet; t, triplet.

target saponin (1 mg) dissolved in methanol (3 mL). After 5 h of stirring at 90 °C, the mixture was cooled to room temperature and diluted with water (20 mL), and the pH value was adjusted to 7.0 by adding an aqueous sodium hydroxide solution. Aliquots (1 mL) were analyzed by means of high-performance anion exchange chromatography using a Dionex ICS-5000 IC (Thermo Fisher Scientific, Waltham, MA, USA) consisting of a Dionex ICS-5000 DP dual pump, a capillary and microbore eluent generator, an AS-AP autosampler, and an ICS-5000 CD type conductivity detector as well as an ICS-5000 EC type electrochemical detector operating in pulsed amperometric detection mode. The detector was equipped with a gold working electrode and a PdH reference electrode supplied by the manufacturer. Data acquisition and instrumental control were completed with Chromeleon software (version 6.80, Thermo Fisher Scientific). Chromatographic separation was performed at 30 °C on a 150 × 0.4 mm CarboPac PA-20 column (Thermo Fisher Scientific) connected to a CarboPac PA-20 guard column (10 × 0.4 mm, Thermo Fisher Scientific). The following gradient was applied: a 10 mM potassium hydroxide solution was run isocratically for 12 min, increasing to 100 mM in 0.1 min and keeping conditions for 4.9 min; thereafter, the gradient was adjusted to the initial 10 mM concentration in 0.1 min and kept for another 19.9 min. After each sample, the column was washed with a sodium hydroxide solution (200 mM) and equilibrated with potassium hydroxide solution (10 mM) for 10 min prior to injection. Chromatography was performed with an injection volume of 0.4 μL and a flow rate of 8 μL/min. Using carbohydrates as reference chemicals, glucose and rhamnose were unequivocally identified as the sugars in saponins **1**−**4**.

Syntheses of Avenanthramides (5−12). With some modifications, avenanthramides **5**−**12** were synthesized according to the literature.[27] Anthranilic acid (21 mmol) and Meldrum's acid (21 mmol) were mixed in dried toluene (15 mL) and refluxed for 4 h. After cooling to room temperature, a saturated $NaHCO_3$ (15 mL) solution was added, followed by stepwise addition of concentrated HCl (10 mL). The white precipitate was filtered, washed with water, and dried at 100 °C to obtain 2-(2-carboxyacetyl)aminobenzoic acid

DOI: 10.1021/acs.jafc.6b04995
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 4. ^{1}H NMR (500 MHz, Pyridine-d_6) and ^{13}C NMR Signals (125 MHz, Pyridine-d_6) of the Aglycone Moiety of Saponins 3 and 4

	3[a,b]				4[a,b]			
position	δ_C	HSQC	δ_H	M [J, Hz]	δ_C	HSQC	δ_H	M [J, Hz]
1	38.5	[CH_2]	1.03−1.12	d	38.5	[CH_2]	1.03−1.12	d
			1.84−1.92	dt			1.84−1.92	dt
2	30.7	[CH_2]	1.56−1.65	o	30.8	[CH_2]	1.57−1.63	o
			1.88−1.95	o			1.89−1.95	o
3	79.4	[CH]	3.54−3.63	o	79.4	[CH]	3.56−3.62	o
4	39.6	[CH_2]	2.25−2.33	t	39.5	[CH_2]	2.25−2.33	t
			2.41−2.48	dd			2.41−2.48	dd
5	141.9	[C]	–		141.9	[C]	–	
6	122.6	[CH]	5.35−5.41	d	122.6	[CH]	5.35−5.41	d
7	33.6[a]	[CH_2]	1.64−1.70	o	33.6[a]	[CH_2]	1.65−1.69	o
			2.01−2.09	o			2.03−2.09	o
8	32.8	[CH]	1.61−1.69	m	32.8	[CH]	1.61−1.69	m
9	51.7	[CH]	0.93−1.01	o	51.7	[CH]	0.93−1.01	o
10	38.0	[C]	–		38.0	[C]	–	
11	22.0	[CH_2]	1.47−1.61a	m	22.0	[CH_2]	1.46−1.63a	m
			1.47−1.61a	m			1.46−1.63a	m
12	40.9	[CH_2]	1.17−1.24	o	40.9	[CH_2]	1.17−1.24	o
			1.75−1.82	o			1.75−1.82	o
13	41.6	[C]	–		41.5	[C]	–	
14	57.7	[CH]	1.10−1.18	m	57.7	[CH]	1.10−1.18	m
15	33.2[a]	[CH_2]	1.20−1.27	o	33.2[a]	[CH_2]	1.19−1.27	o
			1.94−2.00	o			1.95−2.00	o
16	82.1	[CH]	4.43−4.50	ddd	82.1	[CH]	4.43−4.50	ddd
17	63.3	[CH]	1.72−1.80	o	63.3	[CH]	1.72−1.80	o
18	16.6	[CH_3]	0.78−0.85	s	16.6	[CH_3]	0.77−0.85	s
19	19.8	[CH_3]	0.99−1.10	s	19.8	[CH_3]	0.99−1.10	s
20	39.4	[CH]	2.14−2.20	m	39.4	[CH]	2.14−2.20	m
21	15.1	[CH_3]	0.96−1.01	d	15.1	[CH_3]	0.96−1.02	d
22	121.8	[C]	–		121.7	[C]	–	
23	33.2[a]	[CH_2]	1.19−1.27	o	33.2[a]	[CH_2]	1.20−1.28	o
			1.94−2.00	o			1.94−2.01	o
24	33.7[a]	[CH_2]	1.64−1.70	o	33.7[a]	[CH_2]	1.65−1.69	o
			2.01−2.09	o			2.02−2.09	o
25	85.2	[C]	–		85.2	[C]	–	
26	77.6	[CH_2]	3.45−3.50	d	77.6	[CH_2]	3.45−3.50	d
			3.82−3.88	d			3.82−3.88	d
27	24.2	[CH_3]	1.18−1.27	s	24.2	[CH_3]	1.18−1.27	s

[a]Arbitrary numbering according to structures **3** and **4** in Figure 1. [b]Assignments were based on HSQC, HMBC, *J*-res and COSY experiments. d: duplet, m: multiplet signals; o: overlapped with other signals; s: singlet; t: triplet.

(12 mmol; 57% yield) as the intermediate for the synthesis of avenanthramides **8**−**11**. The same protocol was applied to 5-hydroxyanthranilic (21 mmol) and Meldrum's acid (21 mmol) to afford 5-hydroxy-2-(2-carboxyacetyl)aminobenzoic acid (11 mmol; 52% yield) for further synthesis of avenanthramides **5**−**7** and **12**, respectively.

Pyridine (3 mL) and catalytic amounts of *β*-alanine were added to an equimolar mixture of 2-(2-carboxyacetyl)aminobenzoic acid (2.5 mmol) and either *p*-hydroxybenzaldehyde, 3,4-dihydroxybenzaldehyde, vanillin, or syringaldehyde, 2.5 mmol each) to give avenanthramides **8**−**11**. For synthesis of **5**−**7** and **12**, pyridine (3 mL) and *β*-alanine were added to 5-hydroxy-2-(2-carboxyacetyl)-aminobenzoic acid (2.5 mmol) and syringaldehyde (2.5 mmol), respectively. After heating for 110 min at 115 °C under reflux, the mixture was cooled with ice and acidified with concentrated HCl (3 mL). The precipitated avenanthramides (**5**−**12**) were filtered, washed with water, dried in a laboratory oven at 100 °C, and purified by means of preparative HPLC on a 250 × 21.0 mm, 5 *μ*m, Phenyl-Hexyl Luna column (Phenomenex). Monitoring the effluent at 280 and 340 nm, chromatography was performed using 0.1% formic acid in water (effluent A) and acetonitrile (effluent B) at a flow rate of 19 mL/min and isocratic conditions of 45, 50, and 65% of effluent B, respectively. Collected fractions containing the target compounds were combined and separated from solvent under vacuum. After lyophilization, the avenanthramides **5**−**12** were obtained as white, amorphous powder in purity of >98% (HPLC-DAD, ^{1}H NMR) and their structures verified by means of LC-MS and NMR spectroscopy. UV−vis, LC-MS, and 1D/2D-NMR data of *N*-(3′,4′-dihydroxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid (avenanthramide 2c, **5**), *N*-(4′-hydroxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid (avenanthramide 2p, **6**), and *N*-(4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid (avenanthramide 2f, **7**) were identical to those recorded for the compounds isolated above from oats.

*N-(4′-Hydroxy-(E)-cinnamoyl)anthranilic acid (avenanthramide 1p), **8** (Figure 1):* UV−vis (ACN/0.1% HCOOH, 70/30 v/v) λ_{max} = 236 nm, 332 nm; LC-MS (ESI$^-$) *m/z* 282.0 ([$C_{16}H_{13}NO_4$ − H]$^-$), 238.0 ([$C_{15}H_{13}NO_2$ − H]$^-$), 161.8 ([$C_9H_9NO_2$ − H]$^-$); MS/MS (DP = −85 V) *m/z* (%) 282.0 (100), 238.0 (43), 210.0 (15), 161.8 (8), 144.0 (22), 92.0 (27); LC-TOF-MS *m/z* 282.0804 ([M − H]$^-$, measured), calculated for [$C_{16}H_{13}NO_4$ − H]$^-$ *m/z* 282.0766; ^{1}H

DOI: 10.1021/acs.jafc.6b04995
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

Table 5. ^{1}H NMR (500 MHz, Pyridine-d_6) and ^{13}C NMR Signals (125 MHz, Pyridine-d_6) of the Sugar Moieties of Saponins 3 and 4

	3[a,b]				4[a,b]			
position	δ_C	HSQC	δ_H	M [J, Hz]	δ_C	HSQC	δ_H	M [J, Hz]
β-D-GlcI (at C-26)								
1′	105.0	[CH]	4.26−4.32	d [J = 7.7]	105.0	[CH]	4.27−4.33	d [J = 7.7]
2′	75.3	[CH]	3.15−3.29	dd	75.3	[CH]	3.17−3.27	dd
3′	77.8	[CH]	3.30−3.39	dd	77.8	[CH]	3.33−3.39	dd
4′	71.6	[CH]	3.23−3.32	dd	71.7	[CH]	3.25−3.32	dd
5′	76.2	[CH]	3.34−3.41	o	76.2	[CH]	3.36−3.42	o
6′	62.8	[CH_2]	3.63−3.70	o	62.8	[CH_2]	3.63−3.68	o
			3.82−3.91	o			3.83−3.90	o
β-D-GlcII (at C-3)								
1″	100.4	[CH]	4.48−4.53	d [J = 7.7]	100.5	[CH]	4.48−4.54	d [J = 7.6]
2″	78.6	[CH]	3.38−3.44	t	78.6	[CH]	3.39−3.45	t
3″	77.9	[CH]	3.62−3.68	o	77.9	[CH]	3.62−3.68	o
4″	81.1	[CH]	3.52−3.57	t	81.0	[CH]	3.52−3.57	t
5″	76.2	[CH]	3.36−3.41	o	76.2	[CH]	3.36−3.41	o
6″	61.9	[CH_2]	3.63−3.70	o	61.9	[CH_2]	3.63−3.68	o
			3.82−3.91	o			3.80−3.90	o
β-D-GlcIII (at C-4GlcII)								
1‴	104.2	[CH]	4.43−4.48	d [J = 7.9]	104.7	[CH]	4.36−4.42	d [J = 7.8]
2‴	74.4	[CH]	3.33−3.46	t	75.1	[CH]	3.15−3.26	dd
3‴	87.8	[CH]	3.52−3.59	t	78.0	[CH]	3.33−3.40	t
4‴	69.9	[CH]	3.33−3.43	o	71.4	[CH]	3.25−3.33	o
5‴	77.8	[CH]	3.38−3.42	o	78.0	[CH]	3.32−3.39	o
6‴	62.4	[CH_2]	3.78−3.90	o	62.4	[CH_2]	3.63−3.68	o
			3.78−3.90	o			3.80−3.90	o
α-L-Rha (at C-2GlcII)								
1⁗	102.1	[CH]	5.20−5.26	bs [J = 2.5]	102.0	[CH]	5.22−5.26	bs [J = 2.5]
2⁗	72.2	[CH]	3.85−3.90	dd	72.2	[CH]	3.87−3.91	dd
3⁗	72.4	[CH]	3.63−3.68	dd	72.4	[CH]	3.62−3.68	dd
4⁗	73.9	[CH]	3.34−3.43	o	73.9	[CH]	3.39−3.45	o
5⁗	69.7	[CH]	4.09−4.15	m	69.7	[CH]	4.10−4.15	m
6⁗	17.9	[CH_3]	1.19−1.26	d	17.9	[CH_3]	1.18−1.27	d
β-D-GlcIV (at C-3GlcIII)								
1⁗′	105.3	[CH]	4.51−4.58	d [J = 7.7]				
2⁗′	75.5	[CH]	3.23−3.29	t				
3⁗′	76.2	[CH]	3.37−3.42	o				
4⁗′	71.7	[CH]	3.23−3.32	o				
5⁗′	77.8	[CH]	3.33−3.39	o				
6⁗′	62.6	[CH_2]	3.59−3.70	o				
			3.78−3.91	o				

[a]Arbitrary numbering according to structures **3** and **4** in Figure 1. [b]Assignments were based on HSQC, HMBC, *J*-res, and COSY experiments. d, doublet; m, multiplet signals; o, overlapped with other signals; bs, broad singlet; t, triplet.

NMR (400 MHz, MeOD-d_4) δ 6.54 [d, 1H, J = 15,6 Hz, H−C(8′)], 6.83 [d, 2H, J = 8.6 Hz, H−C(3′/5′)], 7.15 [m, 1H, H−C(5)], 7.50 [d, 2H, J = 8.6 Hz, H−C(2′/6′)], 7.57 [m, 1H, H−C(4)], 7.61 [d, 1H, J = 15.6 Hz, H−C(7′)], 8.11 [dd, 1H, J = 8.0, 1.6 Hz, H−C(6)], 8.69 [dd, 1H, J = 8.5, 0.7 Hz, H−C(3)]; ^{13}C NMR (100 MHz, MeOD-d_4) δ 116.8 [C(3′/5′)], 117.4 [C(1)], 119.2 [C(8′)], 121.5 [C(3)], 123.9 [C(5)], 127.4 [C(1′)], 131.0 [C(2′/6′)], 132.6 [C(6)], 135.2 [C(4)], 142.4 [C(2)], 143.7 [C(7′)], 161.0 [C(4′)], 167.2 [C(9′)], 171.5 [C(7)].

N-(3′,4′-Dihydroxy-(E)-cinnamoyl)anthranilic acid (avenanthramide 1c), ***9*** *(Figure 1):* UV−vis (ACN/0.1% HCOOH, 70:30 v/v) λ_{max} = 244 nm, 340 nm; LC-MS (ESI$^-$) m/z 298.0 ([$C_{16}H_{13}NO_5$ − H]$^-$), 161.6 ([$C_8H_5NO_3$ − H]$^-$), 134.8 ([$C_8H_8O_2$ − H]$^-$); MS/MS (DP = −65 V) m/z (%) 298.0 (34), 161.8 (54), 134.8 (100), 117.8 (22), 91.8 (36); LC-TOF-MS m/z 298.0733 ([M − H]$^-$, measured), calculated for [$C_{16}H_{13}NO_5$ − H]$^-$ m/z 298.0715; ^{1}H NMR (400 MHz, MeOD-d_4) δ 6.47 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.80 [d, 1H, J = 8.2 Hz, H−C(5′)], 6.99 [dd, 1H, J = 2.0, 8.3 Hz, H−C(6′)], 7.08 [d, 1H, J = 2.0 Hz, H−C(2′)], 7.14 [m, 1H, H−C(5)], 7.52 [d, 1H, J = 15.6 Hz, H−C(7′)], 7.56 [m, 1H, H−C(4)], 8.10 [dd, 1H, J = 8.0, 1.5 Hz, H−C(6)], 8.68 [dd, 1H, J = 8.5, 0.7 Hz, H−C(3)]; ^{13}C NMR [100 MHz, MeOD-d_4) δ 115.2 [C(2′)], 116.6 [C(5′)], 117.5 [C(1)], 119.2 [C(8′)], 121.6 [C(3)], 122.7 [C(6′)], 123.9 [C(5)], 127.9 [C(1′)], 132.6 [C(6)], 135.2 [C(4)], 142.8 [C(2)], 144.0 [C(7′)], 146.8 [C(3′)], 149.3 [C(4′)], 167.2 [C(9′)], 171.5 [C(7)].

N-(4′-Hydroxy-3′-methoxy-(E)-cinnamoyl)anthranilic acid (avenanthramide 1f), ***10*** *(Figure 1):* UV−vis (ACN/0.1% HCOOH, 70/30 v/v) λ_{max} = 240 nm, 340 nm; LC-MS (ESI$^-$) m/z 312.0 ([$C_{17}H_{15}NO_5$ − H]$^-$), 267.8 ([$C_{16}H_{15}NO_3$ − H]$^-$), 251.8 ([$C_{16}H_{15}NO_2$ − H]$^-$); MS/MS (DP = −90 V) m/z (%) 312.0 (100), 267.8 (41), 251.8 (68), 175.0 (10), 133.0 (43), 91.8 (20); LC-TOF-MS m/z 312.0868 ([M − H]$^-$, measured), calculated for [$C_{17}H_{15}NO_5$ − H]$^-$ m/z 312.0872; ^{1}H NMR (400 MHz, MeOD-d_4) δ 3.93 [s, 3H, H−C(10′)], 6.59 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.83 [d, 1H, J = 8.1 Hz, H−C(5′)], 7.11 [dd, 1H, J = 8.1, 1.9 Hz, H−C(6′)], 7.15 [m, 1H, H−C(5)], 7.25 [d, 1H, J = 1.9 Hz, H−C(2′)], 7.57 [m,

DOI: 10.1021/acs.jafc.6b04995
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1H, H−C(4)], 7.61 [d, 1H, J = 15.6 Hz, H−C(7′)], 8.12 [dd, 1H, J = 8.0, 1.5 Hz, H−C(6)], 8.69 [dd, 1H, J = 8.5, 0.7 Hz, H−C(3)]; ^{13}C NMR [100 MHz, MeOD-d_4) δ 56.5 [C(10′)], 111.6 [C(2′)], 116.5 [C(5′)], 117.7 [C(1)], 119.5 [C(8′)], 121.6 [C(3)], 123.9/124.0 [C(5/6′), may be interchangeable], 127.9 [C(1′)], 132.6 [C(6)], 135.2 [C(4)], 142.7 [C(2)], 144.0 [C(7′)], 149.4 [C(3′)], 150.4 [C(4′)], 167.2 [C(9′)], 171.6 [C(7)].

N-(4′-Hydroxy-3′,5′-dimethoxy-(E)-cinnamoyl)-anthranilic acid (avenanthramide 1s), ***11*** *(Figure 1):* UV−vis (ACN/0.1% HCOOH, 70:30 v/v) λ_{max} = 244 nm, 344 nm; LC-MS (ESI$^-$) m/z 341.8 ([$C_{18}H_{17}NO_6$ − H]$^-$), 297.8 ([$C_{17}H_{17}NO_4$ − H]$^-$), 281.8 ([$C_{16}H_{13}NO_4$ − H]$^-$), 266.8 ([$C_{16}H_{13}NO_3$ − H]$^-$); MS/MS (DP = −85 V) m/z (%) 341.8 (100), 297.8 (35), 281.8 (29), 266.8 (32), 143.8 (27), 120.8 (45); LC-TOF-MS m/z 342.1025 ([M − H]$^-$, measured), calculated for [$C_{18}H_{17}NO_6$ − H]$^-$ m/z 342.0978; ^{1}H NMR (400 MHz, MeOD-d_4) δ 3.91 [s, 6H, H−C(10′/11′)], 6.61 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.95 [s, 2H, H−C(2′/6′)], 7.15 [m, 1H, H−C(5)], 7.57 [m, 1H, H−C(4)], 7.60 [d, 1H, J = 15.6 Hz, H−C(7′)], 8.11 [dd, 1H, J = 8.0, 1.5 Hz, H−C(6)], 8.69 [d, 1H, J = 8.2 Hz, H−C(3)]; ^{13}C NMR [100 MHz, MeOD-d_4) δ 56.9 [C(10′/11′)], 106.8 [C(2′/6′)], 117.6 [C(1)], 119.9 [C(8′)], 121.6 [C(3)], 123.9 [C(5)], 126.9 [C(1′)], 132.6 [C(6)], 135.2 [C(4)], 139.3 [C(4′)], 142.7 [C(2)], 144.2 [C(7′)], 149.5 [C(3′/5′)], 167.1 [C(9′)], 171.6 [C(7)].

N-(4′-Hydroxy-3′,5′-dimethoxy-(E)-cinnamoyl)-5-hydroxyanthranilic acid (avenanthramide 2s), ***12*** *(Figure 1):* UV−vis (ACN) λ_{max} = 236 nm, 348 nm; LC-MS (ESI$^-$) m/z 358.0 ([$C_{18}H_{17}NO_7$ − H]$^-$), 314.0 ([$C_{17}H_{17}NO_5$ − H]$^-$), 298.0 ([$C_{17}H_{17}NO_4$ − H]$^-$), 133.8 ([$C_7H_5NO_2$ − H]$^-$); MS/MS (DP = −80 V) m/z (%) 358.0 (100), 314.0 (42), 298.0 (15), 190.8 (20), 159.8 (36), 133.8 (24); LC-TOF-MS m/z 358.0926 ([M − H]$^-$, measured), calculated for [$C_{18}H_{17}NO_7$ − H]$^-$ m/z 358.0927; ^{1}H NMR (400 MHz, MeOD-d_4) δ 3.90 [s, 6H, H−C(10′/11′)], 6.59 [d, 1H, J = 15.6 Hz, H−C(8′)], 6.93 [s, 2H, H−C(2′/6′)], 7.00 [dd, 1H, J = 2.9, 9.0 Hz, H−C(4)], 7.52 [d, 1H, J = 2.9 Hz, H−C(6)], 7.54 [d, 1H, J = 15.5 Hz, H−C(7′)], 8.46 [d, 1H, J = 8.9 Hz, H−C(3)]; ^{13}C NMR (101 MHz, MeOD-d_4) δ 56.8 [C(10′/11′)], 106.7 [C(2′/6′)], 118.3 [C(6)], 119.4 [C(8′)], 121.7 [C(4)], 123.4 [C(3)], 127.9 [C(1′)], 134.7 [C(2)], 139.2 [C(4′)], 143.4 [C(7′)], 149.5 [C(3′/5′)], 154.2 [C(5)], 166.6 [C(9′)], 171.8 [C(7)].

High-Performance Liquid Chromatography (HPLC). Preparative analyses of fractions II-C and II-D was done on a HPLC apparatus (Jasco, Groß-Umstadt, Germany) consisting of two PU-2087 Plus pumps and an MD 2010 Plus photodiode array detector as well as a Sedex LT-ELSD detector model 85 (Sedere, Alfortville, France) and an Rh 7725i type Rheodyne injection valve (Rheodyne, Bensheim, Germany). The split ratio was set to 1 mL/min for the ELSD detector. Data acquisition was executed by means of Chrompass 1.9. (Jasco).

UPLC/Time-of-Flight Mass Spectrometry (UPLC/TOF-MS). High-resolution mass spectra were measured on a Waters Synapt G2-S HDMS time-of-flight mass spectrometer (Waters, Manchester, UK) coupled to an Acquity UPLC Core system (Waters, Milford, MA, USA). Data acquisition and interpretation were performed by using MassLynx software v4.1 SCN 851 (Waters, Milford, USA) and the tool "Elemental Composition".

High-Performance Liquid Chromatography/Tandem Mass Spectrometry (LC-MS/MS). For structure elucidation, mass and product ion spectra were acquired on an API 4000 Q Trap triple-quadrupole/linear ion trap mass spectrometer (AB Sciex, Darmstadt, Germany). The isolated fractions were dissolved in a mixture of acetonitrile/water (50:50, v/v) and directly introduced into the mass spectrometer by flow infusion using a syringe pump. For electrospray ionization, the ion spray voltage was set at −4500 V in the negative mode and at 5500 V in the positive mode. Both quadrupoles operated at unit mass resolution, and nitrogen served as a curtain gas (25 psi) and as a turbo gas (425 °C). Fragmentation of the pseudo molecular ions [M + H]$^+$ or [M − H]$^-$ into specific product ions was induced by collision with nitrogen (4.5 × 10^{-5} Torr). Data acquisition and instrumental control were performed with Analyst 1.5.1 (AB Sciex).

HPLC-MS/MS analysis of taste compounds was performed on a 150 × 2.0 mm, 5 μm, Luna C18 PhenylHexyl column (Phenomenex) linked to the mass spectrometer operated in the multiple reaction monitoring mode (MRM) in negative electrospray ionization. Using acetonitrile containing 0.1% formic acid as solvent A and 0.1% formic acid in water as solvent B, chromatography was performed using the following gradient at a flow rate of 0.3 mL/min: 0 min, 40% A (1 min isocratically); in 1 min to 70% A (2 min isocratically); in 2 min to 100% A (3 min isocratically); within 2 min back to 40% A (3 min isocratically). Nitrogen served as curtain gas (25 psi), nebulizer gas (55 psi), and turbo gas (45 °C). Fragmentation of the pseudo molecular ions [M − H]$^-$ into specific product ions was induced by collision with nitrogen (4.5 × 10^{-5} Torr). The following mass transitions were recorded for the taste compounds using the declustering potential (DP), collision energy (CE), and collision cell exit potential (CXP) given in parentheses: **5**, m/z 314.2 → 178.0 (DP/CE/CXP −65/−16/−11); **6**, m/z 298.1 → 253.9 (DP/CE/CXP − 50/−22/−15); **7**, m/z 328.0 → 283.8 (DP/CE/CXP −95/−24/−19); **8**, m/z 282.0 → 238.0 (DP/CE/CXP − 85/−24/−7); **9**, m/z 298.0 → 161.7 (DP/CE/CXP −65/−16/−11); **10**, m/z 312.0 → 251.7 (DP/CE/CXP −90/−34/−17); **11**, m/z 342.0 → 298.1 (DP/CE/CXP − 85/−24/−9); **12**, m/z 358.1 → 314.1 (DP/CE/CXP −80/−24/−9).

Nuclear Magnetic Resonance Spectroscopy (NMR). One- and two-dimensional ^{1}H and ^{13}C NMR spectra were acquired on a 500 MHz Bruker Avance III, equipped with a triple-resolution cryo probe (TCI) (Bruker, Rheinstetten, Germany). Samples were dissolved in methanol-d_4 containing 0.03% trimethylsilane (TMS) or pyridine-d_5. The chemical shifts are referenced to the TMS or the solvent signal (pyridine-d_5: ^{1}H 7.22 ppm; ^{13}C 123.87 ppm). TOPSPIN version 2.1 (Bruker) was used for data processing. ^{1}H, ^{13}C, 135DEPT, COSY, J-RESOLVED, ROESY, HSQC, and HMBC spectroscopies were recorded using standard pulse sequences of the Bruker library. Interpretation of the obtained spectra was performed with MestReNova 8.1.0-11315. (Mestrelab Research, Santiago de Compostela, Spain).

■ RESULTS AND DISCUSSION

A freshly prepared suspension of oat flour, imparting a typical astringent and bitter taste, was evaluated by means of a taste profile analysis. Therefore, a trained sensory panel was asked to rate the intensities of the taste modalities bitter, sweet, sour, salty, umami, and astringent on a linear 5-point intensity scale. Bitter and astringent notes were rated with the highest taste intensities of 2.0 and 1.1, respectively (Table 1). A comparatively low intensity was reported for sweetness (0.9), whereas sour (0.3), umami (0.2), and salty taste (0.1) were almost not detectable. To gain a first insight into the hydrophobicity of the compounds imparting the typical bitter and astringent orosensation, oat flour was extracted with solvents of different polarities.

Solvent Fractionation of Oat Flour. Oat flour was extracted sequentially with n-hexane, methanol/water (70:30, v/v), and methanol to give the hexane solubles (fraction I), the methanol/water extractables (fraction II), the methanol extractables (fraction III), and the nonsoluble residue (fraction IV) after solvent separation under vacuum and lyophilization. Taste profile analysis of the individual fractions, each dissolved in water in its "natural" concentration, revealed no taste activity for fraction IV, thus indicating an exhaustive solvent extraction of taste compounds from oat flour (Table 1). Highest sensory scores were reported for bitterness (2.6) and astringency (1.2) in fraction II, whereas fractions I and III exhibited lower bitter intensities; for example, fractions I and III showed astringency scores of 1.0 and 0.7, respectively (Table 1). Aimed at identifying the key molecules inducing the bitter and astringent taste in oat flour, the most taste-active fraction II was subjected to a sensory-guided fractionation.

DOI: 10.1021/acs.jafc.6b04995
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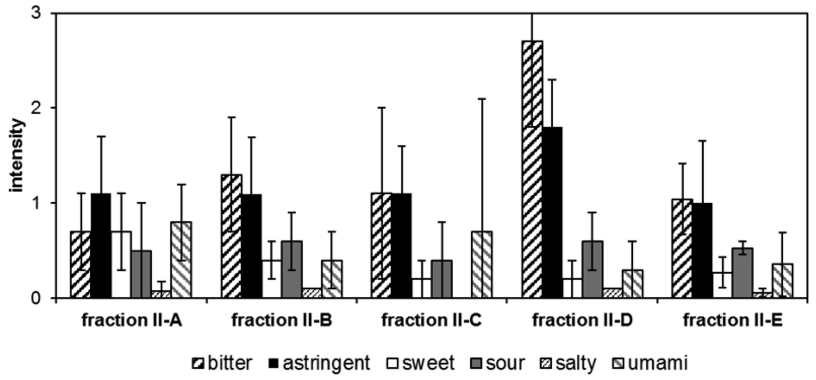

Figure 2. Taste profile analysis of SPE fractions II-A–II-E isolated from oat flour. Sensory data are given as the mean of triplicates; error bars indicate the 95% confidence interval of the arithmetical mean.

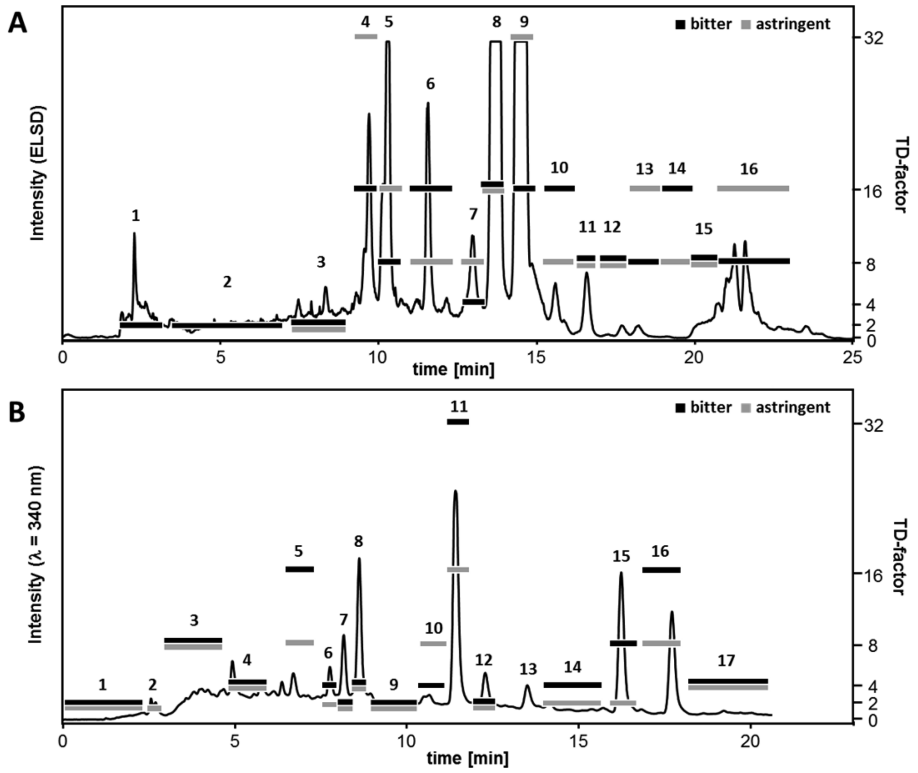

Figure 3. RP-HPLC chromatograms of SPE-fractions II-D (A) and II-C (B) and taste dilution (TD) factors of subfractions II-D-1–II-D-16 and II-C-1–II-C-17.

Sensory-Guided Separation of Oat Fraction II. To sort out the bitter and astringent nonvolatiles from the bulk of tasteless or less active compounds present in fraction II, the methanol/water solubles isolated from oat flour were separated by means of solid phase extraction on an RP-18 material using water, methanol/water mixtures, and methanol as eluents to afford the five subfractions II-A, II-B, II-C, II-D, and II-E. After solvent evaporation under vacuum, the individual subfractions were taken up in water in their "natural" concentration ratios and, then, evaluated in their taste profile (Figure 2). The bitter and astringent sensation of fraction II-D was rated with the highest scores of 2.7 and 1.8, respectively, whereas bitterness and astringency of all other fractions were evaluated with lower intensities ranging between 0.7 and 1.3.

Identification of Taste Compounds in Fraction II-D. To identify the phytochemicals imparting the bitter taste of the most taste-active fraction II-D, this fraction was separated by means of preparative RP-HPLC/ELSD to give 16 subfractions, namely, II-D-1–II-D-16 (Figure 3A), which were collected separately, freed from solvent under vacuum, and used for a TDA. To achieve this, the subfractions were taken up in water in their "natural" concentrations, stepwise 1+1 diluted with water and, then, presented in order of ascending concentrations to a trained sensory panel who was asked to determine the TD factors by means of the half-tongue test.[22−24] Fractions II-D-4–II-D-6, II-D-8–II-D-10, and II-D-14 were evaluated with the highest TD factors for bitterness as well as astringency (Figure 3A). As subfractions II-D-6 and II-D-14 were too unstable to enable an unequivocal identification and subfraction II-D-10 was found to contain the same key compounds as subfraction II-D-9 by carry-over, the following experiments focused on the structure determination of the key taste compounds in subfractions II-D-4, II-D-5, II-D-8, and II-D-9, respectively.

DOI: 10.1021/acs.jafc.6b04995
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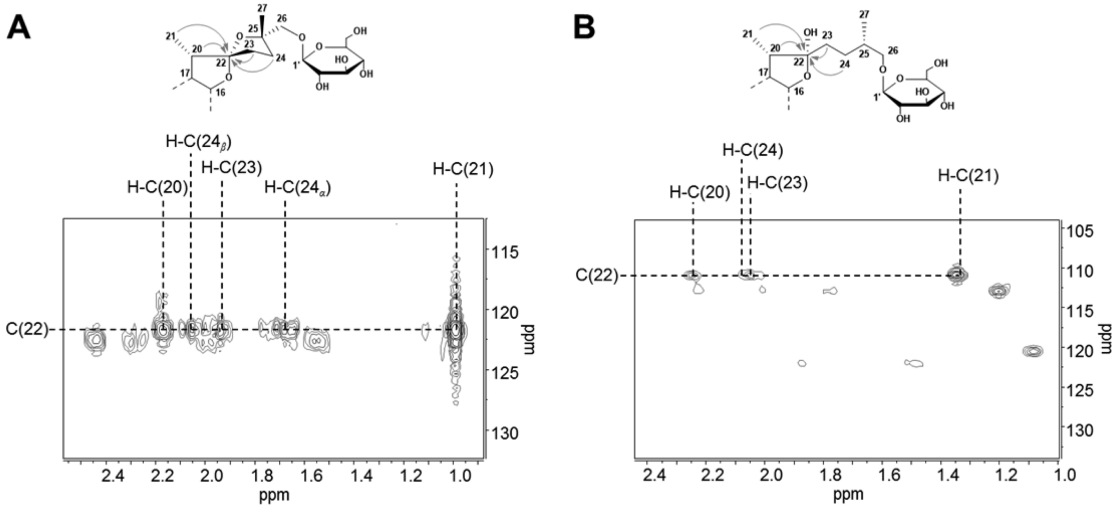

Figure 4. Excerpt of HMBC spectra of the purified saponins **4** (A) and **2** (B).

Preparative RP-HPLC of subfractions II-D-4 and II-D-5, followed by rechromatography, revealed two bitter compounds, **1** and **2** (Figure 1), as amorphous powders. Preliminary separation by means of TLC and spraying with *p*-anisaldehyde/sulfuric acid gave intense green spots suggesting the presence of saponine structures in compounds **1** and **2**.[26] LC-TOF-MS analysis revealed *m/z* 1225.5867 as pseudomolecular ion ($[M - H]^-$) for **1**, indicating a molecular formula of $C_{57}H_{94}O_{28}$. Furthermore, fragment ion peaks with *m/z* 1063.5 ($[M - Glc - H]^-$), 901.4 ($[M - 2Glc - H]^-$), and 755.4 ($[M - 2Glc - Rha - H]^-$) were observed in the negative ESI mode, thus demonstrating the presence of at least two hexose and one deoxyhexose moieties in the saponin. For unequivocal identification of the glycosidically bound carbohydrates, aliquots of the isolates were hydrolyzed with aqueous trifluoroacetic acid, followed by high-performance ion chromatography to D-glucose and L-rhamnose in a ratio of 4:1 for saponin **1** and 3:1 for saponin **2**. The identity of the monosaccharides was confirmed by comparison of their retention times with those found for the corresponding reference compounds as well as by cochromatography.

The ^{13}C NMR spectrum for **1** displayed a total of 57 signals resonating between 16.7 and 141.1 ppm (Tables 2 and 3); 27 of those signals were assigned to the aglycone moiety, and the four signals at 37.4 (C(10)), 41.0 (C(13)), 111.0 (C(22)), and 141.1 ppm (C(5)) were assigned as quarternary carbons by means of an HSQC experiment. The ^1H NMR spectrum revealed the presence of two tertiary methyl groups at 0.90 (H−C(18)) and 1.06 ppm (H−C(19)), three secondary methyl groups at 1.00 (H−C(27)), 1.35 (H−C(21)), and 1.77 ppm (H−C(6_{Rha})), and one olefinic proton at 5.30 ppm (H−C(6)). Moreover, five anomeric carbohydrate protons were observed and assigned as one α-configured rhamnopyranoysl proton (6.24 ppm) showing a coupling constant of 2.2 Hz and four glucopyranosyl moieties (4.83, 4.95, 5.10, and 5.29 ppm) with coupling constants of 7−8 Hz, thus indicating a β-configuration. The bidesmosidic saponin structure was disclosed by means of an HMBC experiment showing long-range correlation between carbon C(26) resonating at 75.5 ppm and the anomeric proton H−C(1_{GlcI}) observed at 4.83 ppm, as well as between carbon atom C(3) at 78.4 ppm and the proton H−C(1_{GlcII}). Full assignment of the remaining carbohydrate atoms and unequivocal identification of the interglycosidic linkage between the monomers were accomplished by means of COSY, HMBC, and ROESY experiments. For example, interglycosidic homonuclear coupling of vicinal protons could be allocated by means of a COSY experiment, homonuclear correlation between the anomeric proton and the protons at C(3_{Glc}) and C(5_{Glc}) was observed in the ROESY spectrum, and heteronuclear $^2J,^3J$ couplings were observed between C(2_{GlcII}) and H−C(1_{Rha}), between C(4_{GlcII}) and H−C(1_{GlcIII}), and between C(3_{GlcIII}) and H−C(1_{GlcIV}), respectively.

The germinal protons H−C(26_a) and H−C(26_b) observed at 3.58−3.66 and 3.92−3.98 ppm matched the data reported for (25*R*)-configured saponins (3.63 and 3.96 ppm).[14,28] As the germinal protons H−C(26_a) and H−C(26_b) of (25*S*)-configured furostanol saponins were reported to resonate at 3.46 and 4.07 ppm,[29,30] the carbon atom C(25) of the furostanol saponin **1** was assigned to be (*R*)-configured. In addition, the methyl group H−C(27), resonating at 0.98−1.02 ppm, supports the (25*R*)-configuration of **1**. By combined analysis of COSY, HSQC, HMBC, DEPT, and *J*-RES data, the aglycone for saponin **1** could be assigned to proto-diosgenin.[31] Taking all MS and 1D/2D-NMR spectroscopic data into consideration, the structure of the bitter compound isolated from fraction II-D-4 was identified as 3-(*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl(1→4)]-β-D-glucopyranosid)-26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol (**1**, Figure 1). Although the (25*S*)-epimer of **1** has been reported as trigoneoside XIIIa in fenugreek seeds of *Trigonella foenum-graecum* L.,[32] the (25*R*)-congener has to the best of our knowledge not yet been reported in literature.

Mass spectrometric analysis of the bitter compound **2**, purified from fraction II-D-5, revealed *m/z* 1063.5334 as the pseudomolecular ion ($[M - H]^-$), thus indicating a molecular formula of $C_{51}H_{84}O_{23}$. The MS daughter ions observed with *m/z* 901.4 ($[M - Glc - H]^-$), 755.1 ($[M - Glc - Rha - H]^-$) and 593.3 ($[M - 2Glc - Rha - H]^-$) as well as the ^1H NMR resonance signals at 4.82, 4.96, 5.14, and 6.25 ppm demonstrated only one rhamnose and three glucose moieties in saponin **2**, but the same aglycone as found for the furostanol saponin **1** (Tables 2 and 3). The HMBC spectrum revealed the aglycone glycosylation by the observed correlation between the proton H−C(1_{GlcII})) at 4.96 ppm and the carbon atom C(3) at

DOI: 10.1021/acs.jafc.6b04995
J. Agric. Food Chem. XXXX, XXX, XXX−XXX

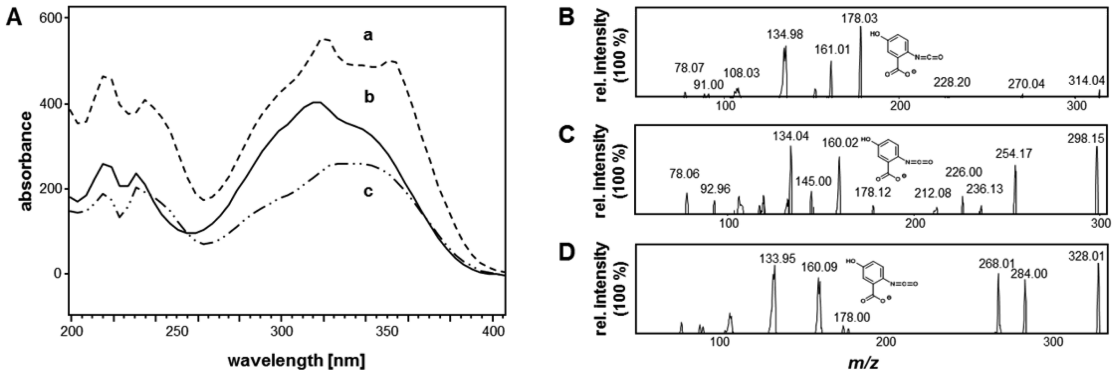

Figure 5. UV−vis spectra (A) of isolated fractions II-C-11 (a), II-C-15 (b) and II-C-16 (c) and MS/MS spectra of compounds **5** (B), **6** (C), and **7** (D).

78.4 ppm, as well as by the heteronuclear connectivity between H−C(1_{GlcII}), resonating at 4.82 ppm, and C−26, observed at 75.5 ppm. Comparison of 1D/2D-NMR data of saponin **2** with those recorded for **1** confirmed the lack of the hexose moiety bound to C(3_{GlcIII}) in **1**. In addition, the resonance signal of C(3_{GlcIII}) was upfield shifted to 81.4 ppm (**2**) when compared to saponin **1** (88.6 ppm). Comparison of spectroscopic data with those observed for **1** and with those reported for structurally related derivatives in the literature[14,28−30,33] revealed a (25*R*)-configuration of saponin **2** and allowed its structure determination as 3-(*O*-*α*-L-rhamnopyranosyl(1→2)-[*β*-D-glucopyranosyl(1→4)]-*β*-D-glucopyranosid)-26-*O*-*β*-D-glucopyranosyl-(25*R*)-furost-5-ene-3*β*,22,26-triol (**2**, Figure 1). To the best of our knowledge, this bitter saponin isolated from *A. sativa* L. has not yet been described elsewhere.

Purification of the key bitter and astringent phytochemicals from fractions II-D-8 and II-D-9 revealed the target compounds **3** and **4**, respectively (Figure 1). LC-TOF-MS analysis exhibited *m*/*z* 1223.5695 and 1061.5169 as the pseudomolecular ions ($[M - H]^-$) and an elemental composition of $C_{57}H_{92}O_{28}$ and $C_{51}H_{82}O_{23}$, respectively. Additional LC-MS/MS experiments, performed in the ESI^+ mode, revealed *m*/*z* 1063.5 ($[M - Glc + H]^+$), 901.4 ($[M - 2Glc + H]^+$), 739.4 ($[M - 3Glc + H]^+$), 593.3 ($[M - 3Glc - Rha + H]^+$), and 431.3 ($[M - 4Glc - Rha + H]^+$) as main daughter ions of compound **3**. Whereas compound **4** exhibited exactly the same daughter ions at *m*/*z* 901.4, 739.4, 593.3, and 431.3, the lower mass by 162 Da indicated one hexose moiety less than found for compound **3**. These findings were well in line with the nuatigenin-type steroidal saponins avenacosides B and A, which had been previously isolated from oat.[10,34−36]

^{1}H NMR data indicated the identical aglycone for both saponins **3** and **4**. In contrast to the furostanol-type saponin skeletons of **1** and **2**, analysis of the HMBC signals recorded for **3** and **4** demonstrated a spirostanol-type aglycone as reported earlier.[34] Due to the spiro ring in compounds **3** and **4**, carbon C(22) observed at 121.7 ppm and C(25) resonating at 85.2 ppm were shifted to lower fields. Long-range correlation signals for C(22) in compounds **3** and **4** (Figure 4A) and, in comparison, in compounds **1** and **2** (Figure 4B) are displayed in the excerpts of the HMBC experiments. In addition, the bidesmosidic structure of saponins **3** and **4** was confirmed by the observed heteronuclear connectivity between glycosidic proton H−C(1_{GlcI}) at 4.30 ppm and carbon atom C(26) at 24.2 ppm, as well as the correlation between H−C(1_{GlcII}) at 4.51 ppm and carbon atom C(3) at 79.4 ppm. High-performance ion chromatographic analysis of monosaccharides obtained after acidic hydrolysis revealed glucose and rhamnose moieties as part of the sugar units. ^{1}H and ^{13}C NMR data (Tables 4 and 5) were well in line with those published previously.[34] Whereas Pecio et al.[34] referenced the signals of C(12) and C(13) to be 41.6 and 40.3 ppm, we assigned carbon C(12) and C(13) as shifted to 40.9 and 41.6 ppm, respectively. This alignment is endorsed by previous studies showing that carbon C(12) in steroidal saponins is high-field shifted.[14,32,37,38] With all spectroscopic data taken into consideration, the chemical structure of the bitter and astringent molecules in fractions II-D-8 and II-D-9 were determined to be nuatigenin-3-*O*-(*α*-L-rhamnopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→3)-*β*-D-glucopyranosyl-(1→4)]-*β*-D-glucopyranoside)-26-*O*-*β*-D-glucopyranoside (**3**), and nuatigenin-3-*O*-(*α*-L-rhamnopyranosyl-(1→2)-[*β*-D-glucopyranosyl-(1→4)]-*β*-D-glucopyranoside)-26-*O*-*β*-D-glucopyranoside (**4**, Figure 1). Although both saponins have been earlier reported in the seeds, grains, and leaves of *A. sativa* L.,[10,34,36,39,40] avenacosides B (**3**) and A (**4**) have not yet been demonstrated to contribute to the bitter taste of oats.

Identification of Taste Compounds in Fraction II-C. As HPLC analysis of the bitter and astringent fractions II-B and II-C showed more quantitative rather than qualitative differences in composition, the most intense taste compounds were located in fraction II-C by means of RP-HPLC-TDA (Figure 3B). Among the 17 subfractions collected, by far the highest TD factor of 32 was found for bitter fraction II-C-11, followed by fractions II-C-5, II-C-16, and II-C-5, exhibiting a bitter impression with TD factors of 16 and 8, respectively. Next to their bitter taste, these fractions exhibited a puckering astringent orosensation evaluated with TD factors between 8 and 16. The following experiments were focused on the identification of the key taste compounds in fractions II-C-5, II-C-11, II-C-15, and II-C-16.

The bitter and astringent compounds detected in subfractions II-C-11, II-C-15 and II-C-16 gave similar UV−vis absorbance spectra exhibiting maxima between 310 and 360 nm (Figure 5). LC-TOF-MS analysis showed pseudomolecular ions ($[M - H]^-$) with *m*/*z* 314.0669, 298.0716, and 328.0851 for the target taste compounds **5**−**7** in fractions II-C-11, II-C-15, and II-C-16, thus indicating the incorporation of one nitrogen atom in each molecule. Furthermore, MS/MS analysis demonstrated losses of 136, 120, and 150 amu, respectively, to result in a characteristic fragment ion with *m*/*z* 178 for all three molecules **5**−**7** (Figure 5), matching well the phenylisocyanate fragment ion reported to be formed upon MS analysis from the 5-hydroxyanthranilic acid moiety of avenanthramides.[41] To further confirm the structure of the

DOI: 10.1021/acs.jafc.6b04995
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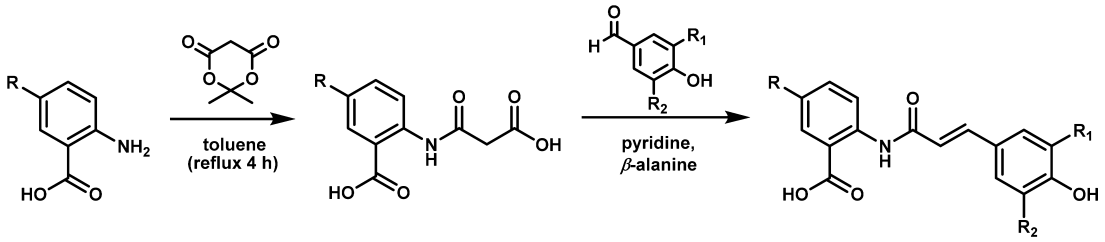

avenanthramide	R	R_1	R_2
5	OH	OH	H
6	OH	H	H
7	OH	OCH_3	H
8	H	H	H
9	H	OH	H
10	H	OCH_3	H
11	H	OCH_3	OCH_3
12	OH	OCH_3	OCH_3

Figure 6. Reaction sequence used for synthesis of avenanthramides **5**−**12**.

suggested 5-hydroxyanthranilic acid structures, 1D- and 2D-NMR experiments were performed.

The ^{1}H NMR spectra of each compound revealed three proton signals showing ortho and meta couplings detected in the aromatic region at 6.9 and 8.4 ppm, which were attributed to H−C(4) and H−C(3) in a COSY experiment. Furthermore, the olefinic protons H−C(8′) and H−C(7′), resonating at 6.5 and 7.5 ppm, demonstrated a coupling constant of 15.7 Hz, indicating the characteristic (*E*)-configuration for cinnamic acid derivatives. The ^{1}H NMR of compound **7** revealed an additional singlet at 3.9 ppm with an intensity of three protons, thus indicating a methoxy group (C−10′) in the chemical structure. With these data taken into consideration, the structures of these bitter and astringent compounds were assumed to be *N*-(3′,4′-dihydroxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid (**5**), *N*-(4′-hydroxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid (**6**), and *N*-(4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid (**7**) as shown in Figure 1. Coined avenanthramides 2c, 2p, and 2f, these compounds have been previously reported as phytochemicals in oats.[42,43]

To confirm the proposed structures and to study the presence of other avenanthramides reported in oats,[42,44] reference compounds of compounds **5**−**7** as well as additional avenathramides (**8**−**12**) were chemically synthesized (Figure 6). Following a literature protocol with some modifications,[27] malonylation of anthranilic acid or 5-hydroxyanthranilic acid with Meldrum's acid, followed by a condensation reaction with benzaldehyde derivatives, gave the crude targeted amides, which were purified by RP-18 chromatography to afford the individual avenanthramides 2c (**5**), 2p (**6**), 2f (**7**), 1p (**8**), 1c (**9**), 1f (**10**), 1s (**11**), and 2s (**12**) in a high purity of >98%. Consequently, the identities of **5**−**12** were verified by means of UV−vis, LC-MS/MS, and NMR experiments.

To validate the presence of the avenanthramides in oat flour, an oat extract was analyzed by LC-MS/MS-MRM using characteristic mass transitions of the individual target compounds **5**−**12**. With the exception of **11** and **12** (data not shown), all other avenanthramides (**5**−**10**) have been detected in the oat extract and unequivocally confirmed by comparing retention times with the reference compounds as well as by cochromatography (Figure 7).

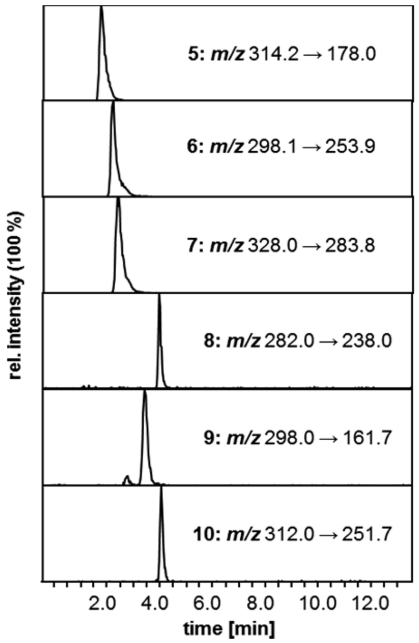

Figure 7. HPLC-MS/MS (ESI$^-$) analysis of phytochemicals **5**−**12**.

Oral Threshold Concentrations of Oat Saponins and Avenanthramides. Prior to sensory evaluation, the purity of the taste compounds was confirmed by means of HPLC-ELSD and LC-MS. To determine the recognition threshold concentrations of the bitter taste and astringent orosensation of the saponins (**1**−**4**) and avenanthramides (**5**−**12**), aqueous solutions (pH 6.4) of the target compounds were evaluated by a trained sensory panel using the half-tongue test.[22,23]

The orosensory threshold concentrations of the avenanthramides **5**−**12** were strongly dependent on their chemical structure and ranged from 38 to 135 μmol/kg for astringency and from 60 to 170 μmol/kg for bitterness (Table 6). The lowest bitter taste threshold of 60 μmol/kg was found for avenanthramide 2p (**6**), showing a *para*-hydroxylated cinnamic acid moiety. Additional hydroxylation (**5**) or methoxylation (**7**) in meta position of the cinnamic acid resulted in somewhat higher orosensory threshold concentrations; for example,

DOI: 10.1021/acs.jafc.6b04995
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Table 6. Human Recognition Threshold Concentrations of Taste Compounds 1−12

taste compound[b]	taste threshold[a] (μmol/kg)	
	astringency	bitterness
3-(*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→3)-β-D-glucopyranosyl(1→4)]-β-D-glucopyranosid)-26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol (**1**)	3 ± 2	4 ± 2
3-(*O*-α-L-rhamnopyranosyl(1→2)-[β-D-glucopyranosyl(1→4)]-β-D-glucopyranosid)-26-*O*-β-D-glucopyranosyl-(25*R*)-furost-5-ene-3β,22,26-triol (**2**)	3 ± 1	9 ± 21
avenacoside B (**3**)	4 ± 12	6 ± 19
avenacoside A (**4**)	3 ± 2	7 ± 20
avenanthramide 2c (**5**)	55 ± 31	78 ± 38
avenanthramide 2p (**6**)	45 ± 17	60 ± 15
avenanthramide 2f (**7**)	135 ± 37	170 ± 55
avenanthramide 1p (**8**)	70 ± 48	96 ± 48
avenanthramide 1c (**9**)	56 ± 2	130 ± 2
avenanthramide 1f (**10**)	98 ± 40	113 ± 25
avenanthramide 1s (**11**)	82 ± 21	101 ± 21
avenanthramide 2s (**12**)	38 ± 23	81 ± 65

[a]Taste threshold concentrations were determined in aqueous solution (pH 6.4) containing 1 (saponins **1**−**4**) or 2% ethanol (avenanthramides **5**−**12**). [b]Structures of compounds are given in Figure 1.

avenanthramide 2f (**7**), exhibiting a ferulic acid moiety, showed bitter and astringency thresholds of 170 and 135 μmol/kg, respectively. With the exception of avenanthramide 2f, the 5-hydroxyanthranilic acid amides, such as 2c (**5**) and 2p (**6**), with thresholds of 60 and 78 μmol/kg were found with higher taste activities when compared to anthranilic acid amides such as avenanthramides 1p (**8**), 1c (**9**), and 1f (**10**), showing bitter taste threshold concentrations of 96, 130, and 113 μmol/kg, respectively.

Sensory evaluation of the saponins (**1**−**4**) revealed astringency and bitter taste at much lower concentrations when compared to the avenanthramides; for example, bitter taste thresholds were found to range between 4 and 9 μmol/kg (Table 6). Among the saponins, the furostanol saponin **1** imparted the lowest threshold concentrationa of 3 and 4 μmol/kg for astringency and bitter taste, respectively.

In conclusion, sensory-guided fractionation of oat flour extracts and chemical synthesis led to the identification of the saponins **1**−**4** as well as the avenanthramides **5**−**10** as the most intense bitter and astringent phytochemicals. Aimed at demonstrating their relative contribution to the overall off-taste of oat flour, quantitative studies are currently in progress and will be published elsewhere.

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Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are thankful to Daniela Günzkofer for her excellent technical assistance. We are grateful to General Mills Inc. for supporting this research project.

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