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Avenanthramides in Oats (*Avena sativa* L.) and Structure−Antioxidant Activity Relationships

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Eight avenanthramides, amides of anthranilic acid (**1**) and 5-hydroxyanthranilic acid (**2**), respectively, and the four cinnamic acids *p*-coumaric (**p**), caffeic (**c**), ferulic (**f**), and sinapic (**s**) acid, were synthesized for identification in oat extracts and for structure−antioxidant activity studies. Three compounds (**2p**, **2c**, and **2f**) were found in oat extracts. As assessed by the reactivity toward 1,1-diphenyl-2-picrylhydrazyl (DPPH), all avenanthramides except **1p** showed activity. Initially, the antioxidant activity of the avenanthramides decreased in a similar order as for the corresponding cinnamic acids, that is: sinapic > caffeic > ferulic > *p*-coumaric acid. The avenanthramides derived from **2** were usually slightly more active than those derived from **1**. All avenanthramides inhibited azo-initiated peroxidation of linoleic acid. **1c** and **1s** were initially the most effective compounds. The relative order of antioxidant activities was slightly different for the DPPH and the linoleic acid assays run in methanol and chlorobenzene, respectively.

KEYWORDS: ***Avena sativa*; avenanthramide; caffeic acid; cinnamic acid; *p*-coumaric acid; dehydrodicinnamic acid dilactone; DPPH; ferulic acid; radical scavengers; sinapic acid**

INTRODUCTION

Autoxidation of polyunsaturated fatty acids lowers the nutritional value of foods. However, it has been known for a long time that antioxidants, by protecting against rancidity and by conserving color, flavor, and texture, can considerably increase the shelf life of foods (*1*). Antioxidants are currently receiving increasing interest as they also may be expected to prevent membrane damage, age-related deterioration, heart disease, and cancer (*1*, *2*). Living organisms are equipped with enzymatic as well as nonenzymatic antioxidant protection systems. Plants are rich in nonenzymatic antioxidants such as carotenoids, vitamins C and E, and various phenolic compounds (*3*).

Phenols exert their antioxidant activity by acting primarily as hydrogen atom donators (*3*), thereby inhibiting the propagation of radical chain reactions. In addition, some phenols act as metal ion chelators. Their potential as antioxidants is dependent on the number and arrangement of hydroxyl groups, as well as the nature of the substituents in the ring structures. Some antioxidants are known to act in a synergistic fashion. Regeneration of α-tocopherol by vitamin C and the protection of β-carotene by α-tocopherol are some examples of synergisms (*4*).

Oats have been considered to be a good antioxidant source for a long time (*5*). The most well-known compounds with antioxidant activity are tocopherols and tocotrienols and derivatives of benzoic and cinnamic acids and aldehydes (*6*). More recently, a group of amides unique to oats, and trivially named avenanthramides, has been found.

Six avenanthramides derived from anthranilic acid (**1**), 5-hydroxyanthranilic acid (**2**), or 5-hydroxy-4-methoxyanthranilic acid (**3**) and *p*-coumaric (**p**), caffeic (**c**) or ferulic (**f**) acid, namely, **1p**, **1f**, **2p**, **2c**, **2f**, and **3f** (**Figure 1**), have been isolated and identified in oat grains (*7*−*10*). In addition, Collins and Mullin (*8*) reported **1c** and three avenanthramides derived from 4-hydroxyanthranilic acid, but structural data were not presented. Sinapic acid (**s**) is found in oats (*11*), but it is not known if avenanthramides derived from this acid are present in the plant. However, chromatographic and spectroscopic data indicate that additional unidentified avenanthramides are contained in oat extracts (*7*, *9*, *10*).

2p, **2c**, and **2f** have been shown to be related to the fresh taste of oat products (*12*) and may thus function as antioxidants protecting against rancidification. So far, two avenanthramides, **2f** and **3f**, have been investigated in vitro for antioxidant activity,

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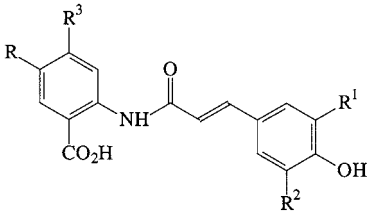
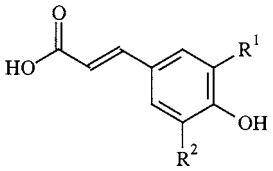


Avenanthramide	R	R^1	R^2	R^3
1p	H	H	H	H
1c	H	OH	H	H
1f	H	OCH_3	H	H
1s*	H	OCH_3	OCH_3	H
2p	OH	H	H	H
2c	OH	OH	H	H
2f	OH	OCH_3	H	H
2s*	OH	OCH_3	OCH_3	H
3f	OH	OCH_3	H	OCH_3



Cinnamic acid	R^1	R^2
p-coumaric acid	H	H
caffeic acid	OH	H
ferulic acid	OCH_3	H
sinapic acid	OCH_3	OCH_3

Figure 1. Avenanthramides and derivatives of cinnamic acid referred to in this study. *Compounds not previously reported from oats.

and both were found to be active. In a linoleic acid system, **3f** exerted an antioxidant activity ~3 times higher than that of **2f** (*9*). As the avenanthramides are present in small amounts in oats and have similar physical and chemical properties, they are difficult to isolate in sufficient quantities. To study the structure−antioxidant activity relationships of the avenanthramides and to identify them in crude oat extracts, synthesis was required.

The aim of this work was to synthesize avenanthramides, to study the structure−antioxidant activity relationships of avenanthramides and their corresponding cinnamic acids in one hydrophilic and one lipophilic system, to study the combined antioxidant effects of avenanthramides, and to identify avenanthramides in oat extracts.

MATERIALS AND METHODS

Chemicals. *p*-Coumaric, caffeic, ferulic, sinapic acids, their corresponding aldehydes, and anthranilic, 5-hydroxyanthranilic, and linoleic acids were all purchased from Aldrich-Chemie (Steinheim, Germany). 1,1-Diphenyl-2-picrylhydrazyl (DPPH) was purchased from Sigma Chemical Co. (St. Louis, MO) and DL-α-tocopherol from Hoffman-La Roche (Basel, Switzerland). Solvents used were of reagent or HPLC grade or dried and distilled according to standard procedures.

Oat Samples. Samples of two covered cultivars, Sang and Salo (1997), were provided from Cerealia AB (Järna, Sweden), and four naked cultivars, Bikini, Bullion, Neon, and Ripon (1998), were provided from Svalöf Weibull AB (Svalöv, Sweden). The four naked cultivars were grown at the same site, whereas the two samples of the covered cultivars were taken from a commercial batch.

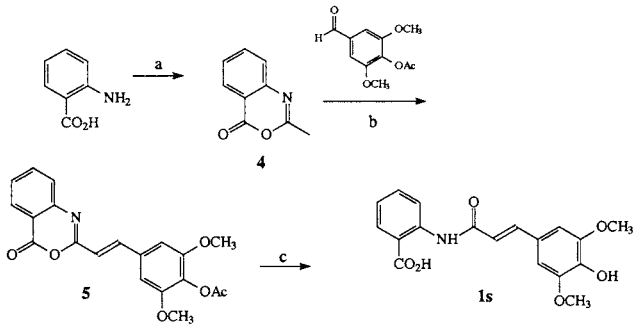


Figure 2. Synthetic route to avenanthramides exemplified by **1s**: (a) $(Ac)_2O$, reflux; (b) TsOH, toluene, reflux; (c) (*i*) Na_3PO_4 (aq), (*ii*) HCl.

Instrumentation. ^1H NMR (400 MHz) and ^{13}C NMR (100.5 MHz) spectra were recorded on a Varian Unity 400 instrument. Chemical shifts (δ) are reported in parts per million, using residual solvent as internal standard. Thin-layer chromatography was performed on Merck HF-254 silica gel plates with pentane/ethyl acetate in various proportions as mobile phases. IR spectra were recorded on a Perkin-Elmer 1600 FT-IR spectrometer and UV spectra and the DPPH measurements on a Shimadzu UV-2101PC spectrophotometer. For stimulated autoxidation of linoleic acid, HPLC was performed on a Waters 600 E instrument equipped with an autoinjector (Gilson 233 XL with thermostated sample rack), a photodiode array detector (Waters 996), and a Millennium 32 chromatography data system. For detection of avenanthramides in oat extracts a Hewlett-Packard series 1100 HPLC instrument, equipped with a photodiode array detector and ChemStation version 05.01 software, was used.

Synthesis. A modified version of the method described by Mayama et al. (*13*) was used. The procedure for synthesis of avenanthramides is exemplified by the synthesis of **1s** (**Figure 2**).

4-Acetoxy-3,5-dimethoxybenzaldehyde. Syringaldehyde (5.0 g, 27.5 mmol) was added to a solution of acetic anhydride (3.0 g, 29.5 mmol) in pyridine (30 mL). The mixture was refluxed for 2 h. The solvent was evaporated to give the title compound (6.1 g, 99%) as white crystals. The crude product was used in the next step without further purification.

2-Methylbenzoxazin-4-one (4). 2-Aminobenzoic acid (7.0 g, 51 mmol) was treated with acetic anhydride (60 mL) under reflux for 4 h to give a dark red solution (*14*). The solvent was evaporated to give the title compound (7.95 g, 97%) as white crystals. The crude product was used in the next step without further purification.

2-[2′-(4″-Acetoxy-3″,5″-dimethoxyphenyl)ethenyl]benzoxazin-4-one (5). To a solution of **4** (0.748 g, 4.65 mmol) and 4-acetoxy-3,5-dimethoxybenzaldehyde (1.11 g, 4.93 mmol) in dry toluene (50 mL) was added *p*-toluenesulfonic acid (0.085 g, 0.49 mmol). The mixture was refluxed for 48 h under an atmosphere of dry nitrogen and allowed to cool to room temperature. The solvent was evaporated. The yellow crystals were dissolved in CH_2Cl_2 (200 mL). Water (200 mL) was added and the organic layer separated. The water was extracted with CH_2Cl_2 (2 × 100 mL). The organic phase was dried over $MgSO_4$ and filtered, and the solvent was evaporated to give a quantitative yield of the title compound: ^1H NMR ($CDCl_3$) δ 8.21 (ddd, J = 7.9, 1.6, 0.6 Hz, 1H), 8.01 (ddd, J = 8.1, 7.2, 1.6 Hz, 1H), 7.77 (d, J = 16.0 Hz, 1H), 7.59 (ddd, J = 8.1, 1.2, 0.6 Hz, 1H), 7.50 (ddd, J = 7.9, 7.2, 1.2 Hz, 1H), 6.83 (s, 2H), 6.74 (d, J = 16.0 Hz, 1H), 3.87 (s, 6H), 2.35 (s, 3H); ^{13}C NMR (DMSO) 168.5, 159.2, 157.1, 152.5, 147.0, 141.7, 136.6, 132.9, 130.4, 128.7, 128.3, 126.9, 119.0, 116.9, 104.5, 56.1, 20.4; IR ν_{max} cm^{-1} 1745, 1598, 1222, 1129; UV (EtOH) λ_{max} 206, 331.

N-[4-Hydroxy-3,5-dimethoxy-(E)-cinnamoyl]anthranilic Acid (1s). Crude product **5** (0.457 g, 1.25 mmol) was ring opened and deprotected by refluxing in 0.01 M Na_3PO_4(aq) for 20 h. The resulting red solution was allowed to cool to room temperature. After filtration, the solution was acidified with HCl (0.1 M) to pH 2, and stirred at 0 °C for 20 min to give a yellow precipitate. The product was filtered off. The mother liquor was extracted with ethyl acetate (4 × 150 mL). The solvent was removed to give additional crystals. The crystals were rinsed with a small amount of dilute HCl and filtered off (0.386 g). The crude

product was recrystallized from acetone/water to give the title compound as yellow crystals (0.172 g, 40%).

The NMR data for *N*-[4-hydroxy-(*E*)-cinnamoyl]anthranilic acid (**1p**), *N*-[4-hydroxy-3-methoxy-(*E*)-cinnamoyl]anthranilic acid (**1f**), *N*-[4′-hydroxy-(*E*)-cinnamoyl]-5-hydroxyanthranilic acid (**2p**), *N*-[3′,4′-dihydroxy-(*E*)-cinnamoyl]-5-hydroxyanthranilic acid (**2c**), and *N*-[4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl]-5-hydroxyanthranilic acid (**2f**) were in good agreement with literature data (*7*).

*N-[3,4-Dihydroxy-(E)-cinnamoyl]anthranilic Acid (**1c**).* ^{1}H NMR (DMSO-d_6) δ 11.24 (s, COOH, 1H), 9.53 (s, OH, 1H), 9.13 (s, OH, 1H), 8.58 (dd, J = 8.4, 1.1 Hz, 1H), 7.99 (ddd, J = 7.9, 1.7, 0.4 Hz, 1H), 7.59 (ddd, J = 7.4, 7.4, 1.7 Hz, 1H), 7.44 (d, J = 15.5 Hz, 1H), 7.15 (ddd, J = 7.9, 7.3, 1.2 Hz, 1H), 7.08 (d, J = 2.1 Hz, 1H), 7.00 (ddd, J = 8.1, 2.2, 0.4.Hz, 1H), 6.77 (d, J = 8.2 Hz, 1H), 6.50 (d, J = 15.5 Hz, 1H); ^{13}C NMR (DMSO) δ 169.5, 164.1, 148.0, 145.6, 141.9, 134.0, 131.1, 125.9, 122.6, 121.2, 120.3, 118.4, 116.6, 115.8, 114.6; IR ν_{max} cm^{-1} 3369, 1636, 1221; UV (MeOH) λ_{max} 339; mp 221−230 °C. Anal. Calcd for $C_{16}H_{13}NO_5 \cdot 0.5H_2O$: C, 62.34; H, 4.57; N, 4.54. Found: C, 61.84; H, 4.47; N, 4.23.

*N-[4-Hydroxy-3,5-dimethoxy-(E)-cinnamoyl]anthranilic Acid (**1s**).* ^{1}H NMR (DMSO-d_6) δ 11.29 (s, COOH, 1H), 8.90 (s, OH, 1H), 8.63 (ddd, J = 8.5, 1.2, 0.4 Hz, 1H), 8.0 (ddd, J = 8.0, 1.7, 0.4 Hz, 1H), 7.60 (ddd, J = 7.7, 7.2, 1.8 Hz, 1H), 7.53 (d, J = 15.4 Hz, 1H), 7.15 (ddd, J = 7.9, 7.2, 1.2 Hz, 1H), 7.04 (s, 2H), 6.76 (d, J = 15.4 Hz, 1H), 3.82 (s, 6H); ^{13}C NMR (DMSO) δ 169.4, 164.2, 148.0, 142.4, 141.1, 137.9, 134.0, 131.1, 124.7, 122.6, 120.3, 119.2, 116.5, 106.1, 56.1; IR ν_{max} cm^{-1} 3401, 1609, 1268; UV (MeOH) λ_{max} 232, 344; mp 199−200 °C. Anal. Calcd for $C_{18}H_{17}NO_6 \cdot H_2O$: C, 59.83; H, 5.30; N, 3.88. Found: C, 59.54; H, 5.20; N, 3.45.

*N-[4′-Hydroxy-3′,5′-dimethoxy-(E)-cinnamoyl]-5-hydroxyanthranilic Acid (**2s**).* ^{1}H NMR (DMSO-d_6) δ 10.84 (s, COOH, 1H), 9.59 (s, OH, 1H), 8.86 (s, OH, 1H), 8.38 (d, J = 9.0 Hz, 1H), 7.47 (d, J = 15.4 Hz, 1H), 7.37 (d, J = 3.0 Hz, 1H), 7.02 (dd, J = 9.0, 3.0 Hz, 1H), 7.00 (s, 2H), 6.72 (d, J = 15.4 Hz, 1H), 3.81 (s, 6H); ^{13}C NMR (DMSO) δ 169.1, 163.7, 152.4, 148.0, 141.5, 137.7, 133.0, 124.9, 122.4, 120.9, 119.5, 118.3, 116.5, 106.0, 56.1; IR ν_{max} cm^{-1} 3371, 1608, 1221; UV (MeOH) λ_{max} 226, 346; mp 206 °C. Anal. Calcd for $C_{18}H_{17}NO_7 \cdot 0.5\ H_2O$: C, 58.69; H, 4.93; N, 3.80. Found: C, 58.44; H, 4.87; N, 3.76.

Antioxidant Activity. *DPPH.* A modified method of Brand-Williams et al. (*15*) was used. One hundred microliters of the avenanthramide or cinnamic acid solution (0.50 mM in methanol) and 900 μL of DPPH solution in methanol (0.076 mM) were mixed in a tube (molar ratio = 1:1.4) at the same time as the scanning session was initialized, immediately vortexed, transferred to a cuvette, and placed in the spectrophotometer. The absorbance (A) at 517 nm was recorded during 20 min. The small spontaneous decrease of absorbance of the DPPH solution was investigated by time (t). The linear equation obtained ($A = -0.0087t + 0.7704$; $R^2 = 0.9713$) was used to calculate a start absorbance for each sample. This start value was used to calculate the decrease of absorbance (ΔA) at 2 min for all samples. DL-α-Tocopherol was run as a reference. DL-α-Tocopherol and caffeic and sinapic acids were also mixed with DPPH in an additional molar ratio (1:5.6). All samples were run in duplicates. The lowest absorbance obtained, using pyrogallol as an antioxidant, was 0.01.

Combination Effects. A combination study was performed in the DPPH system described above. The two avenanthramides with the weakest activity (**1p** and **2p**) were excluded, and combination effects of the remaining six avenanthramides were investigated according to a fractional factorial design with 32 experiments and 6 central points. The experiments were divided into two blocks that were analyzed on two separate days. The design allowed all main effects and two factor interactions to be independently estimated. The high (+) and low (−) concentrations of the six avenanthramides were chosen to obtain a similar antioxidant activity contribution after 20 min from each compound at each level (**Table 1**). Narrow intervals of the concentrations were chosen to get linear relations between concentration and effect.

Linoleic Acid. A slightly modified method of Braughler et al. (*16*) was used. Linoleic acid in chlorobenzene (7.5 mL, 36.2 mM) was stirred (1100 rpm) at 42 °C in a 20 mL thermostated reaction vessel. To this solution was added the inhibitor in ethanol (100 μL, 3.2 mM; 40 μM final concentration) by syringe. After 15 min of stirring/equilibration, a thermostated solution of 2,2′-azobis(2,4-dimethylvaleronitrile) (AMVN) in chlorobenzene (0.5 mL, 22.4 mM) was added. Five microliter samples were withdrawn every 10 min (after interruption of the stirring for 30 s) and injected onto a 150 × 3.9 mm i.d., 5 μm Resolve silica 90 Å column (Waters) eluted with hexane/ethanol (90:10) with a flow rate of 1.0 mL/min. After sampling, stirring was immediately resumed. The reaction was followed for at least 70 min. The formation of conjugated diene hydroperoxides (retention times of 2.8−3.2 min) was monitored at 234 nm, and the concentrations were determined by integration using an experimentally determined response factor [a weighed amount of linoleic acid containing a trace of linoleic acid hydroperoxide was allowed to react in $CDCl_3$ with bis[4-(dimethylamino)phenyl]telluride and the conversion to the corresponding telluroxide was determined by integration in the ^{1}H NMR spectrum]. The data obtained during the first 30 min were adjusted to a linear graph, and the slope was used as a measurement of antioxidant activity.

Table 1. Concentrations of the Individual Avenanthramides Used in the Combination Study (Micromolar)[a]

	c (–)[b]	**c** (0)	**c** (+)	**f** (–)	**f** (0)	**f** (+)	**s** (–)	**s** (0)	**s** (+)
1	40	65	90	80	160	240	40	70	100
2	30	65	100	50	130	210	40	70	100

[a] **1** corresponds to anthranilic acid and **2** to 5-hydroxyanthranilic acid; p, c, f, and s correspond to *p*-coumaric, caffeic, ferulic, and sinapic acid, respectively. [b] (–) low concentration; (0) mean concentration; (+) high concentration.

Oat Avenanthramide Extraction and Analysis. Groats and hulls (separated by hand) (5 g) were extracted and analyzed in triplicate according to the method described by Bryngelsson et al. (*17*). Methanol extracts were subjected to HPLC analysis on a 125 × 4 mm i.d., 5 μm reversed phase C-18 column (HP ODS Hypersil) using a mobile phase consisting of two solvents: A, 0.01 M phosphate buffer (pH 2.8) and acetonitrile (95:5, v/v); B, acetonitrile. Samples were run with a linear gradient over 60 min from 0 to 40% B in A. The components were detected at 340 nm. To identify the avenanthramides in the HPLC chromatograms, retention times and UV spectra of the peaks were compared with those of synthetic standards.

Statistical Analysis. Results from analyses of individual avenanthramides and cinnamic acids were statistically evaluated by Tukey's pairwise comparison ($\alpha = 0.05$). Results from the combination study were statistically evaluated by fractional factorial fit. All analyses were conducted using the software Minitab release 11.12 (Minitab Inc., State College, PA).

RESULTS

Synthesis. A modified version of the method of Mayama et al. (*13*) was used for the preparation of avenanthramides (**Figure 2**). 2-Methylbenzoxazin-4-ones derived from anthranilic (**1**) or 5-hydroxyanthranilic acid (**2**) (O-acetylated) were obtained by treatment with acetic anhydride. Subsequent acid-catalyzed aldol reactions with substituted benzaldehydes gave the protected benzoxazinones, and final treatment with phosphate-promoted hydrolysis resulted in the avenanthramides. All compounds were crystalline with colors varying from pale yellow to brown. A novel feature of the modified procedure is the mild phosphate-induced deprotection and ring-opening step. This treatment gave no side reactions, and the crude product was obtained in ∼90% yield (as judged from NMR data). However, optimal conditions for recrystallization were difficult to find, and only 50% of the pure product was obtained. The syntheses of **1c**, **1s**, and **2s** are reported for the first time.

Structure—Antioxidant Activity Relationships. There are several different methods described in the literature for the evaluation of antioxidant activity (*18* and references cited therein). In the present study, two commonly used methods,

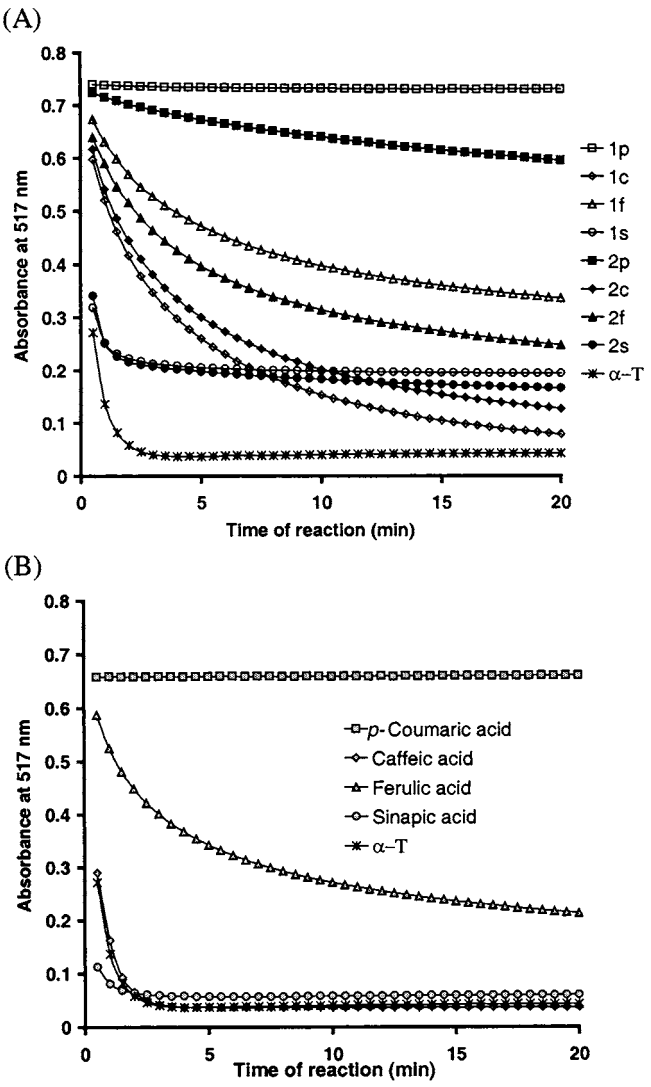


Figure 3. DPPH radical scavenging activity: (A) avenanthramides; (B) cinnamic acids. (Antioxidant/DPPH molar ratio = 1:1.4). α-Tocopherol was used for comparison. Means of duplicates were used to construct the curves. See **Table 1** for abbreviations of avenanthramides.

reactivity toward DPPH (a hydrophilic system) and linoleic acid (a lipophilic system), were chosen. Both methods measure the efficiency of a hydrogen atom transfer from a phenol to a radical.

Figure 3 shows the absorbance of the DPPH radical with time in the presence of the antioxidants tested. In **Figure 3A** it can be seen that all compounds tested except **1p** had activity toward DPPH. **1c**, **2c**, **1s**, and **2s** were the most reactive compounds, followed by **2f**, **1f**, and **2p**. **1s** and **2s**, both derived from sinapic acid, reached their steady state after ~90 s, whereas the other avenanthramides responded much more slowly. During the first 2 min, the cinnamic acid part of the avenanthramides seemed to have the major influence on reactivity, and the activity order was sinapic > caffeic > ferulic > *p*-coumaric acids (**Table 2**). After 20 min, **1c**/**2c** had activity equal to or even higher than that of **1s**/**2s** (**Figure 3A**). Furthermore, the **2** analogues, with their additional OH group, had activities equal to or higher compared to their **1** counterparts. All avenanthramides showed lower activity than α-tocopherol (α-T).

Figure 3B shows that, among cinnamic acids, sinapic and caffeic acids were the most reactive toward DPPH, followed by ferulic acid. *p*-Coumaric acid showed no activity. The initial relative reactivity of the cinnamic acids was similar to that of

Table 2. Antioxidant Activity of Avenanthramides and Cinnamic Acids As Determined by the DPPH Assay[a]

	DPPH assay ($\Delta A_{2\ min}$/0.01 AU)[b]		
	1, avenanthramide	**2**, avenanthramide	cinnamic acid
p	0 ± 0a	3 ± 0a	6 ± 5a
f	16 ± 1b	21 ± 1bcd	24 ± 1bcde
c	31 ± 4e	28 ± 2ce	65 ± 1g
s	51 ± 3f	51 ± 2f	67 ± 0g
control (methanol)	0 ± 0a		
α-tocopherol	65 ± 2g		

[a] See **Table 1** for abbreviations of avenanthramides and cinnamic acids. [b] Figures give the decrease in DPPH absorbance at 2 min. High values indicate high activity. Figures with different letters are significantly different ($p < 0.05$).

the initial reactivity of the corresponding avenanthramides, that is, sinapic > caffeic > ferulic > *p*-coumaric acids (**Table 2**). As was seen for **1s** and **2s**, sinapic acid reached an apparent steady state within 90 s. Caffeic and sinapic acids decolorized the DPPH solution more efficiently than their corresponding avenanthramides, whereas *p*-coumaric and ferulic acids had almost the same reactivity as **1p**/**2p** and **1f**/**2f**, respectively. At the molar ratio of 1:1.4 (antioxidant/DPPH), no differences in reactivity between α-tocopherol and sinapic and caffeic acids could be observed after 2 min. However, at a molar ratio of 1:5.6, the order of reactivity was caffeic acid > α-tocopherol > sinapic acid (data not shown).

Ferulic and sinapic acids as well as **1s** showed higher antioxidant activities than expected. These compounds have only one hydrogen atom available for donation to the DPPH radical, which should result in a final absorbance of ≥0.23 AU. The lower values observed suggest that new compounds, able to donate additional hydrogen atoms, were formed during the course of the experiment. This was also verified by HPLC and ^{1}H NMR analysis of the DPPH/antioxidant mixtures (data not shown). UV and NMR spectra for the main compounds formed were in accordance with literature data for dehydrodiferulic acid dilactone and dehydrodisinapic acid dilactone, respectively, for ferulic and sinapic acids (*19*, *20*). In the DPPH/**1s** mixture, three new compounds were formed, and their identification is currently being undertaken at our laboratories.

In the combination study only one significant ($p = 0.02$) interaction (antagonistic) was found, namely, between **2f** and **1c**. In addition, there was a tendency ($p = 0.06$) toward an antagonistic interaction between **2f** and **1s**. However, it was not possible to decide which avenanthramide was the inhibitor.

Figure 4 shows the concentration of conjugated diene hydroperoxides versus time for the various avenanthramides and their corresponding cinnamic acids during azo-initiated peroxidation of linoleic acid. α-Tocopherol and a control without added antioxidant were included for comparison. The graphs of most of the avenanthramides and the cinnamic acids showed an exponential increase. To compare the activities among compounds, the slopes of the linear graphs obtained during the initial phase of peroxidation (30 min) were used as a measure of antioxidant activity (**Table 3**). The mean regression coefficient values are given in **Table 3**. All avenanthramides inhibited the formation of conjugated diene hydroperoxides. **1c** and **1s** were the most effective compounds and were significantly more active than **1p** and **1f** (**Table 3**). **2s**, **2c**, **2p**, and **2f** all showed similar intermediate activities. After 100 min, though, **2p** and **2f** were the only compounds that still exerted any substantial inhibitory activity (**Figure 4A**). From **Figure 4B**

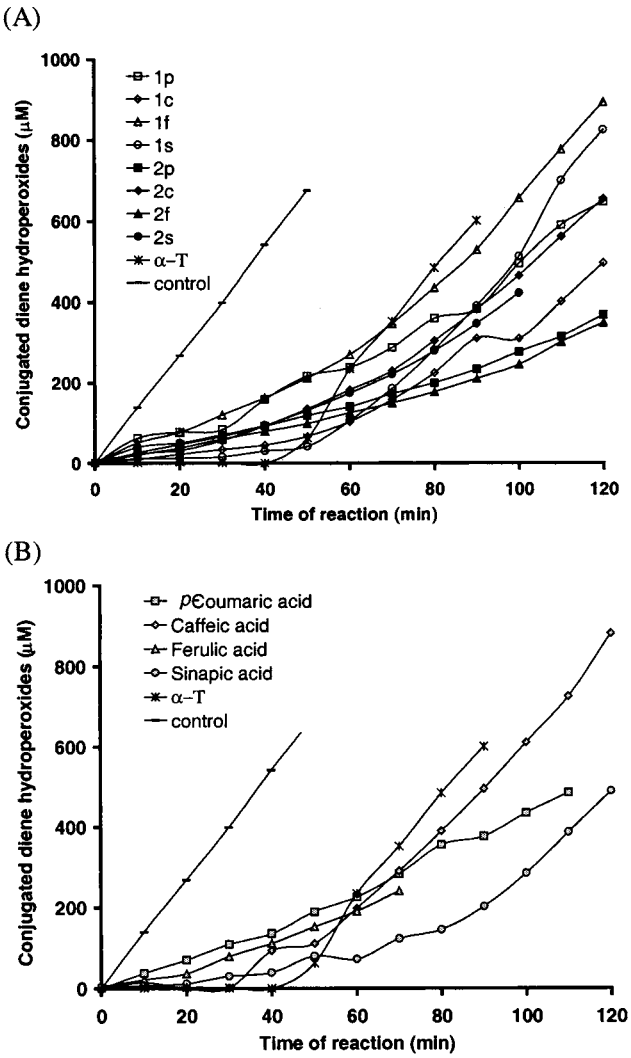


Figure 4. Conjugated diene hydroperoxide concentration versus time: (A) avenanthramides; (B) cinnamic acids. α-Tocopherol was used for comparison. Means of triplicates were used to construct the curves. See **Table 1** for abbreviations of avenanthramides.

and **Table 3** it is evident that sinapic and caffeic acids were the most active antioxidants among the cinnamic acids tested. Ferulic acid showed intermediate activity, whereas *p*-coumaric acid was the poorest. All cinnamic acids were equally as active as their corresponding avenanthramides.

For α-tocopherol, one could clearly distinguish the inhibited phase of peroxidation from the uninhibited one (**Figure 4**). However, for most of the avenanthramides and cinnamic acids the slopes of the lines did not return to that of uninhibited peroxidation. As a consequence, these compounds, compared with α-tocopherol, exerted their antioxidant activity for a longer period of time.

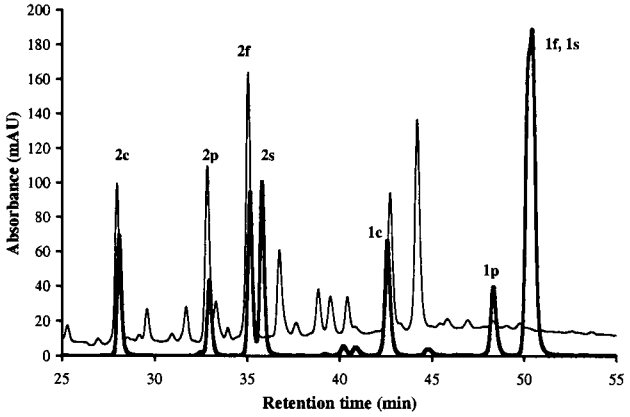


Figure 5. HPLC chromatogram of oat extract (–) and reference compounds (—). See **Table 1** for abbreviations of avenanthramides.

Avenanthramides in Oat Extracts. All oat extracts contained **2p**, **2c**, and **2f** in different concentrations, but **2s** could not be detected (**Figure 5**; **Table 4**). Furthermore, no avenanthramides derived from unsubstituted anthranilic acid (**1**) could be found, either in groats or in hulls. As judged from the UV spectrum, the compounds that eluted at 42 and 48 min in the chromatogram were not **1c** and **1p**, respectively.

DISCUSSION

Antioxidant Activity. In both antioxidant assays, the antioxidant efficiency of cinnamic acids initially decreased in the same order, that is, sinapic > caffeic > ferulic > *p*-coumaric acids. This order of activity is in accordance with earlier findings (*21*−*23*). The same order was also seen for both **1**- and **2**-derived avenanthramides in the DPPH system. However, in the linoleic acid assay this trend was not that apparent. In the DPPH system, the hydroxyl group in **2** seemed to have some influence on the activity, but only at a later stage in the reaction. Due to resonance in the amide bond, the avenanthramides could be expected to possess higher antioxidant activity than the corresponding cinnamic acids. However, this was not the case, and actually the contrary was seen in the DPPH system.

Several hypotheses have been forwarded concerning the fate and further reactions of the phenoxyl radicals resulting from initial hydrogen atom transfer (*15*, *24*). α-Tocopherol is known to trap two radicals ($n = 2$) (*25*). In the linoleic acid assay, the slope of the peroxidation plot of most compounds tested did not return to that corresponding to uninhibited peroxidation. In

Table 3. Antioxidant Activity of Avenanthramides and Cinnamic Acids As Determined by the Linoleic Acid Assay[a]

	linoleic acid assay (μM conjugated diene hydroperoxides/min)[b]					
	1, avenanthramide	R^{2c}	**2**, avenanthramide	R^{2c}	cinnamic acid	R^{2c}
p	3.3 ± 0.5ac	0.819	2.3 ± 0.4acd	0.987	3.5 ± 0.3ac	0.995
f	3.9 ± 0.9a	0.969	2.0 ± 0.4acdf	0.993	2.4 ± 1.6acd	0.696
c	1.0 ± 0.2bde	0.915	1.7 ± 0.2bc	0.912	0.2 ± 0.4bf	0.232
s	0.5 ± 0.2bd	0.510	2.5 ± 1.2aceg	0.896	0.9 ± 0.3bdfg	0.747
control		13.6 ± 0.0h, $R^2 = 1.000$				
α-tocopherol		0.2 ± 0.3bf, $R^2 = 0.572$				

[a] See **Table 1** for abbreviations of avenanthramides and cinnamic acids. [b] Figures give the slope of the conjugated diene hydroperoxide concentration versus time plot during 30 min. Low values indicate high activity. Figures with different letters are significantly different ($p < 0.05$). [c] R^2 = mean regression coefficient values of the slopes.

Table 4. Levels of Avenanthramides in Groats and Hulls from Six Different Oat Cultivars[a]

oat sample	**1p**, **1c**, **1f**, **1s**, **2s**		**2p**		**2c**		**2f**	
	groats	hulls	groats	hulls	groats	hulls	groats	hulls
Sang	nd[b]	nd	13.0	5.1	12.0	nd	7.1	1.6
Salo	nd	nd	22.6	6.9	24.4	2.3	17.2	3.3
Bullion	nd	–[c]	140.0	–	79.0	–	124.5	–
Bikini	nd	–	36.4	–	30.6	–	36.1	–
Ripon	nd	–	36.1	–	24.7	–	21.8	–
Neon	nd	–	31.1	–	30.9	–	20.5	–

[a] All values are given in nmol/g of DM. See **Table 1** for abbreviations of avenanthramides. [b] nd, not detected. [c] –, not analyzed.

the DPPH system, both ferulic and sinapic acids as well as **1s**, which all have only one hydrogen atom for donation, showed higher antioxidant activity than expected. Taken together, these results suggest that new compounds were formed that were capable of quenching radicals. A formation of dehydrodicinnamic acid dilactones in the case of ferulic and sinapic acids is in agreement with previous findings from both enzymatic (oxidoreductases) (*19*, *20*) and nonenzymatic ($FeCl_3$) (*26*) oxidation of hydroxycinnamic acids. The mechanism suggested for this reaction includes an 8,8-coupling of phenoxyl radicals, followed by intramolecular nucleophilic attacks of the two carboxylic acid moieties to form a dilactone (*19*, *20*). Studies of the fate and further reactions of avenanthramides are currently being undertaken.

Results can vary among in vitro antioxidant assays, because of different chemistries and conditions (*18*), and the finding that the relative order of antioxidant activities was slightly different in the two assays studied is not surprising. The "polar paradox", for example, briefly states that nonpolar antioxidants function better in a polar lipid emulsion, whereas polar antioxidants are relatively more effective in bulk lipids (*27*). Many investigations (e.g. refs *28* and *29*) have shown that the rate of hydrogen atom removal from various antioxidants is independent of the nature of the attacking radical (phenoxyl, peroxyl, or aminyl). However, the rate varies substantially with the solvent used. This observed kinetic solvent effect is attributed to hydrogen bonding between antioxidant OH and NH groups, respectively, and hydrogen bond accepting solvents. Some of the antioxidants included in this investigation, namely, those with hydroxy or methoxy groups ortho to the phenolic group, have possibilities for internal hydrogen bonds as well. This effect could of course be different in the aprotic solvent, chlorobenzene, and in the hydrogen bond accepting methanol, thus leading to a slightly different ordering of the compounds with regard to antioxidant efficiency.

Avenanthramides in Oats. The finding that **2p**, **2c**, and **2f** were present in all oat grain extracts is in agreement with previous reports (*7*−*11*, *30*). Collins (*7*) has also identified **1p** and **1f** from oat groat and/or hull extracts, without giving any quantitative data, and Matsukawa et al. (*30*) have detected **1p** in oat seeds (3 nmol/g of fresh weight). In the present study no avenanthramides from the **1** series were found. Nevertheless, the **1**-derived avenanthramides might very well be present in the extracts but in quantities below the detection limit. In the HPLC system used in the present study, the detection limits for avenanthramides were estimated to be 5 and 25 nmol/mL, for hull and groat extracts, respectively (corresponding to 1 and 5 nmol/g, respectively) as the chromatograms contained several interfering peaks. The enzyme hydroxyanthranilate *N*-hydroxycinnmoyltransferase, which catalyzes the biosynthesis of avenanthramides in oat grains, has an affinity for both anthranilic (**1**) and 5-hydroxyanthranilic (**2**) acids, although the highest affinity has been found for 5-hydroxyanthranilic acid (*30*). Furthermore, Dimberg et al. (*9*) have isolated **3f**, which occurs in oats in concentrations below the detection limit of the present methodology. The amounts and composition of avenanthramides may also vary with cultivars and cultivation conditions (*9*, *10*, *31*, *32*).

This work showed that the avenanthramides studied exerted antioxidant activity to various extents and that they function in polar as well as nonpolar systems. They might thereby contribute to the well-known antioxidant capacity of oats.

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