of interest (225 nm). The resulting chromatogram (Figure 1b) showed the presence of both enantiomers **4** and **6** at the same retention times as those in the standard mixture but in unequal amounts, the (*S*)-(−) enantiomer being present at a higher concentration than the (*R*)-(+) ester. The two peaks were integrated with use of Nelson difference and integration programs, which quantified the peak area ratio for the (*S*)-(−) and (*R*)-(+) components as ca 4:1.

The natural form of 2-(4-methoxyphenoxy)propanoic acid, which occurs in Colombian roasted coffee, analyzed as the methyl ester, is therefore predominantly the (*S*)-(−) enantiomer.

Registry No. 4, 4276-74-8; **5**, 117896-90-9; (*R*)-(+)-2-(4-methoxyphenoxy)propanoic acid, 4276-75-9.

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Oat Phenolics: Avenanthramides, Novel Substituted *N*-Cinnamoylanthranilate Alkaloids from Oat Groats and Hulls[1]

F. William Collins

Fractionation of methanolic extracts of oat groats and hulls by anion-exchange chromatography revealed the presence of a series of anionic, substituted cinnamic acid conjugates, trivially named avenanthramides. Two-dimensional thin-layer chromatography (TLC) showed groat extracts contain more than 25 distinct avenanthramides, while hull extracts contained about 20. Some 15 were common to both groat and hull preparations. The substances were purified by repeated column chromatography on Sephadex LH-20, using TLC to monitor purity, and crystallized from aqueous acetone. The complete structures of 10 avenanthramides have been elucidated using ^1H and ^{13}C nuclear magnetic resonance (NMR), mass spectroscopy (MS), ultraviolet absorption spectroscopy (UV), and hydrolytic techniques and confirmed by total synthesis. The physicochemical properties, potential biological activity, and distribution within the oat grain are discussed.

Despite the widespread utilization of oats in the agri-food sector, little work has been done to characterize oat grain phenolic compounds. The structurally dependent involvement of cereal grain phenolics in a wide variety of biochemical and nutritional processes makes a knowledge of the types and amounts of these phenolics extremely valuable. Such information is important in improving the quality of specalized oat-based feed and food ingredients, in developing oat processing technology, in evaluating dietary input data for human and animal nutrition, and as an aid in elucidating mechanisms of disease resistance. An in-depth study of oat grain phenolics is therefore currently being carried out with particular emphasis on the structural elucidation of "bound" forms of the commonly reported hydroxycinnamic acids. The structures and functionality of some of these conjugated phenolics have recently been summarized (Collins, 1986), but a new group of nitrogen-containing constituents, the avenanthramides, have now been detected. The purpose of this paper is to briefly describe their occurrence, isolation, structure, physicochemical properties, and potential biological activity.

EXPERIMENTAL SECTION

Melting points were obtained on a Fisher-Johns apparatus and are uncorrected. Chromatographic separations of extracts and synthetic mixtures were carried out on Sephadex LH-20 (Pharmacia, Canada) using volume-calibrated glass columns and gravity-flow isocratic elution. Solvents used for column chromatography were as follows: I, $CHCl_3$–cyclohexane–MeOH–acetic acid (50:40:5:5); II, $CHCl_3$–cyclohexane–MeOH–acetic acid (50:35:10:5); III, $CHCl_3$–cyclohexane–MeOH–acetic acid (50:30:15:5); IV, $CHCl_3$–cyclohexane–MeOH–acetic acid (50:25:20:5); V, acetone–H_2O–acetic acid (30:65:5).

Chromatographic mobilities were recorded as relative elution values, K', equal to the ratio V_e/V_b, where V_e is the observed elution volume and V_b is the total volume of the packed gel (i.e., bed volume). When small sample application volumes V_c are maintained, relative to V_b (i.e., $V_c/V_b \leq 10\%$), the K' values were found to be independent of the size of the columns used and the process could be scaled up or down as required. Analytical TLC was performed on silica gel plates (Baker-Flex, 1B2-F, Canadian

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Laboratory Supplies Ltd.) using the following solvent systems: A, $CHCl_3$–acetic acid (95:5); B, $CHCl_3$–2-propanol–acetic acid (90:5:5); C, toluene–methyl ethyl ketone–acetic acid (80:14:1); D, acetone–H_2O–acetic acid (30:65:5). Phenolics were detected under UV light before and after plates were sprayed with a 5% solution of ethanolamine in 2-propanol. UV spectra were recorded on Pye Unicam SP 1800 UV spectrometer. Medium-resolution electron impact mass spectra (EIMS) were obtained by direct-inlet solid-probe analysis using a Finnigan MAT 312 or a Finnigan 4500B spectrometer at 70 eV. High-resolution mass spectra (HRMS) were obtained on the Finnigan MAT 312 at nominal resolution of 7500 (solid probe).

Extraction of Oat Grains. Oats (*Avena sativa* L. cult. Sentinel) were dehulled, groats and hulls separately dry ground in a Waring blender, and the resulting powders sieved through a No. 40 U.S. standard-mesh screen (particle size ≤ 425 μm). The following extraction procedure was used for both groats and hulls. The material was slurred in a 5-fold excess (v/w) of aqueous 80% methanol, heated to 55 °C for 15 min with constant stirring, and the cooled overnight at 4 °C. The mixture was reslurried and poured while still cold into a volume-calibrated glass column, fitted with a coarse-porosity scintered glass disk. The column was allowed to equilibrate to room temperature (20 °C) and settle by gravity to give a packed filter bed and a clear yellow-brown supernatant. The supernatant extract was collected by draining the column and eluting the packed bed with five bed volumes of 80% methanol. The bed was then reslurried and the above extraction procedure repeated twice. The eluates from the three extractions were combined, diluted 25% with 2-propanol to reduce foaming and autooxidation, and concentrated in vacuo by rotary evaporation at 30 °C. The resulting syrup (approximately 13% of original weight of groat extracted; 7% for hulls) was dissolved in warm aqueous 50% 2-propanol (55 °C) cooled to 4 °C, reequilibrated to room temperature, and finally filtered by gravity through a coarse-porosity scintered glass filter. The resulting clear amber filtered extract was used in all subsequent analyses.

Group separation of avenanthramides was achieved by anionic fractionation of the extract using ion-exchange column chromatography on Sephadex QAE A-25. The gel was first converted to the formate form and then equilibrated and washed until neutral with 50% 2-propanol. The column was charged with the extract and washed with four bed volumes of 50% 2-propanol to remove neutral and cationic components. The anionic constituents were then eluted with four bed volumes of the acidic solvent, acetone–H_2O–formic acid (60:35:5). The eluate was concentrated to a syrup by rotary evaporation at 30 °C, the last traces of formic acid were removed by repeated washing and reevaporation with 50% 2-propanol, and the final residue was taken up in 50% 2-propanol. Anionic phenolics including the avenanthramides were separated from phospholipids, amino acids, and non-phenolics by a two-stage procedure. First, the zwitterionic constituents were removed by passing the anionic fraction through a Sephadex SP C-25 column in the H^+ form and washing with four bed volumes of 50% 2-propanol. The eluate and washings were combined and concentrated to dryness, and the residue was chromatographed on Sephadex LH-20 by isocratic elution with the solvent acetone–H_2O–acetic acid (40:55:5). After the first two bed volumes of eluate (i.e., $K \leq 2.0$) were discard, the absorbed avenanthramides were then eluted with two bed volumes of 80% aqueous acetone, evaporated to dryness, and taken up in 50% iso-propanol.

Purification of Avenanthramides. To facilitate identification, two-dimensional analytical scale TLC "maps" of oat groat and hull avenanthramides were prepared with solvent systems B and C [see Collins (1986) for representative color photographs of TLC plates]. Individual avenanthramides were purified by repeated preparative-scale column chromatography in solvents I–V using the analytical TLC systems to monitor purity at each step. Purified avenanthramides were crystallized from hot acetone–water.

Synthesis of Avenanthramides. In general, a modification of the procedure of Bain and Smalley (1968) was used. Equimolar quantities of the acyl chloride derivative of the suitably protected aromatic acid and the appropriate free aminobenzoic acid were condensed in the presence of pyridine. Where necessary, the condensation mixture was treated directly with mild alkali to remove the protecting groups and the final product isolated by preparative column chromatography.

Avenanthramide A, N-(4'-Hydroxy-(E)-cinnamoyl)-5-hydroxyanthranilic Acid. 5-Chloro-2-nitrobenzoic acid (2.02 g, 10 mmol; Aldrich Chemical Co.) was refluxed for 24 h in 1.0 N NaOH (50 mL), cooled to room temperature, acidified with HCl, and extracted three times with ethyl acetate. The combined ethyl acetate fraction was evaporated to dryness by rotary evaporation and the residue recrystallized from cold water to give 5-hydroxy-2-nitrobenzoic acid as yellow needles: mp 171 °C; yield 98%. Reduction with a 3-fold excess of $Na_2S_2O_4$ in 2.5% NH_4OH at room temperature for 30 min followed by removal of solvent by rotary evaporation gave a residue containing the sodium bisulfite doulbe salt of 5-hydroxy-2-aminobenzoic acid (5-hydroxyanthranilic acid). The free acid was recovered by desalting this residue on SP-Sephadex H^+ form and QAE-Sephadex formate form columns and recrystallizing from cold water to give pale purple needles: mp 250 °C (dec) (darkens at 235 °C; $C_7H_7NO_3$; $M^{\bullet+}$ 153; UV (MeOH) λ_{max} 222, 246s, 338 nm; UV (MeOH + HCl) λ_{max} 216, 233, 301, 365 nm; UV (MeOH + NaOH) λ_{max} 224, 242s, 338 nm. 4-Acetoxy-(*E,Z*)-cinnamoyl chloride was prepared from 4-hydroxy-(*E,Z*)-cinnamic acid (Aldrich) by acetylation with acetic anhydride (*p*-toluenesulfonic acid catalyst) and treatment of the recrystallized (hot MeOH) 4-acetoxy-(*E,Z*)-cinnamic acid with excess thionyl chloride according to the procedures of Fosdick and Starke (1940). Removal of excess thionyl chloride by repeated rotary evaporation and washing with acetone gave a crude acid chloride containing no detectable free 4-acetoxy-(*E,Z*)-cinnamic acid. The crud acid chloride was found suitable for subsequent reactions and was used without further purification. A solution of 153 mg (1 mmol) of 5-hydroxy-2-aminobenzoic acid in 10 mL of pyridine was added to the dried residue corresponding to (2 mmol) 4-acetoxy-(*E,Z*)-cinnamoyl chloride dissolved in 10 mL of acetone. The mixture was warmed briefly on a sand bath (100 °C) and cooled to room temperature, and the solvents were removed by repeated evaporation and washing with 80% aqueous acetone on a rotary evaporator. The residue was dissolved in acetone–acetic acid–H_2O (80:10:10) and allowed to stand overnight to ensure hydrolysis of any unreacted acyl chloride and any cyclodehydration products. After removal of the solvent by rotary evaporation, the final residue was refluxed in MeOH–H_2O–concentrated NH_4OH (50:40:10) for 30 min to remove the acetyl protecting group. After cooling, the solution was evaporated to dryness by rotary evaporation at 30 °C to remove the excess ammonia and the products were chromatographed

in solvent III to give *N*-(4′-hydroxy-(*E*)-cinnamoyl)-5-hydroxy-2-aminobenzoic acid (avenanthramide A; K' = 4.94–5.70, yield 174 mg (58.2%)) and a small amount of the *Z* isomer, avenanthramide A-1 (K' = 4.17–4.94). Crystallization of the *E* isomer from hot acetone–water gave clusters of pale yellow needles: mp 277 °C; $C_{16}H_{13}NO_5$; $M^{\bullet+}$ 299; UV (MeOH) λ_{max} (log ϵ) 220 (4.35), 298s, (4.29), 305s, (4.36), 322 (4.45), 339 (4.45) nm; UV (MeOH + NaOH) λ_{max} (log ϵ) 214 (4.45), 233s, (4.29), 308s, (4.07), 317s, (4.15), 377 (4.56) nm. This synthetic product was found to be identical (EIMS, HRMS: calcd 299.0794, obsd 299.0796; ^{1}H NMR, UV, mp, cochromatography in solvents II, III, and V) with avenanthramide A isolated from oat extracts.

Avenanthramide B, N-(4-Hydroxy-3′-methoxy-(E)-cinnamoyl)-5-hydroxyanthranilic Acid. Crude 4-acetoxy-3-methoxy-(*E*,*Z*)-cinnamoyl chloride was prepared from 4-hydroxy-3-methoxy-(*E*,*Z*)-cinnamic acid (Aldrich) by the same procedure as outlined for 4-acetoxy-(*E*,*Z*)-cinnamoyl chloride. A solution of 153 mg (1 mmol) of 5-hydroxy-2-aminobenzoic acid was condensed with the dried residue corresponding to (2 mmol) 4-acetoxy-3-methoxy-(*E*,*Z*)-cinnamoyl chloride as outlined above for avenanthramide A. After removal of the protecting group with mild alkali, the products were chromatographed in solvents II and III to give *N*-(4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl)-5-hydroxy-2-aminobenzic acid (avenanthramide B; K' = 5.09–6.18 and K' = 2.78–3.29, respectively, yield 182 mg (55.3%)) and a small amount of the *Z* isomer avenanthramide B-1 (K' = 4.38–5.09 and K' = 2.32–2.78, respectively). Crystallization of the *E* isomer from hot acetone–water gave long pale yellow needles: mp 246 °C, $C_{17}H_{15}NO_6$; $M^{\bullet+}$ 329; UV (MeOH) λ_{max} 225, 242, 304s, 334s, 354 nm; UV (MeOH + NaOH) λ_{max} 228, 254s, 306s, 315, 361, 392 nm. This synthetic product was found to be identical (EIMS, ^{1}H NMR, UV, mp, cochromatography in solvents II, III, and V) with avenanthramide B isolated from oat extracts.

Avenanthramide D, N-(4′-Hydroxy-(E)-cinnamoyl)-anthranilic Acid. A solution of 135 mg (1 mmol) of anthranilic acid (Aldrich) was condensed with the dried residue corresponding to (1 mmol) 4-acetoxy-(*E*,*Z*)-cinnamoyl chloride as outlined for avenanthramide A. After deacylation with mild alklai, the products were chromatographed in solvents I and II to give *N*-(4′-hydroxy-(*E*)-cinnamoyl)-2-aminobenzoic acid (avenanthramide D; K' = 6.61–7.40 and 2.82–3.51, respectively, yield 235 mg (83%)) and a small amount of the *Z* isomer, avenanthramide D-1 (K' = 5.69–6.61 and 2.22–2.82, respectively). Crystallization of the *E* isomer from hot acetone–water gave colorless rods: mp 219 °C; $C_{16}H_{13}NO_4$; $M^{\bullet+}$ 283; UV (MeOH) λ_{max} (log ϵ) 218 (4.30), 294s, (4.25), 302s, (4.33), 329 (4.47) nm; UV (MeOH + NaOH) λ_{max} (log ϵ) 213 (4.43), 233s, (4.19), 306s, (4.05), 314 (4.08), 371 (4.51) nm. This synthetic product was found to be identical (EIMS, ^{1}H NMR, UV, mp, cochromatography in solvents II and V) with avenanthramide D from oat extracts.

Avenanthramide E, N-(4′-Hydroxy-3′-methoxy-(E)-cinnamoyl)anthranilic Acid. A solution of 135 mg (1 mmol) of anthranilic acid was condensed with the dried residue corresponding to (1 mmol) 4′-acetoxy-3′-methoxy-(*E*,*Z*)-cinnamoyl chloride as outlined for avenanthramide B. After deacetylation, the products were chromatographed in solvents I and II to give *N*-(4′-hydroxy-3′-methoxy-(*E*)-cinnamoyl)-2-aminobenzoic acid (avenanthramide E; K' = 2.63–3.25 and 1.88–2.31, respectively, yield 225 mg (72%)) and a small amount of the *Z* isomer, avenanthramide E-1 (K' = 2.06–2.53 and 1.42–1.88, respectively). Crystallization of the *E* isomer from hot acetone–H_2O gave pale yellow needles: mp 235 °C; $C_{17}H_{15}NO_5$; $M^{\bullet+}$ 313; UV (MeOH) λ_{max} 211, 236s, 246s, 292s, 304s, 338 nm; UV (MeOH + NaOH) λ_{max} 219, 262s, 301s, 385 nm. This synthetic compound was found to be identical (EIMS, ^{1}H NMR, UV, mp, cochromatography in solvents II and V) with avenanthramide E from oat extracts.

RESULTS AND DISCUSSION

Chromatographic Properties of Avenanthramides. The anion-exchange techniques used in this study were originally developed to quantitatively remove and subsequently recover "free" hydroxycinnamic and benzoic acids from covalently linked or "bound" forms (e.g., esterified). However, it was observed that only traces of ferulic, *p*-coumaric, and several related benzoic acids were recovered in the free acid form from oat extracts, and that these represented relatively minor components in a complex mixture of hitherto unreported acidic phenolics. Chromatographically these novel phenolics formed a distinct group of closely related compounds for which the collective name avenanthramides is proposed. They exhibited unusually high binding affinity toward polyamide TLC and column chromatographic media (nylon 11, 6, and 6.6) as compared to simple hydroxybenzoic acid and cinnamic acids. Once adsorbed on polyamides, elution could only be achieved by strong acid or bases, and the mixtures were not resolved. A silica gel 2-dimensional analytical TLC system was therefore developed, enabling resolution of the avenanthramide mixture from oat groat extracts into at least 40 chromatographically distinct components. Individual avenanthramides appeared colorless to bright yellow in daylight with colors varying from black, gray, and brown, absorbing to dull yellow, dull green, purple, blue, and white fluorescent in long-wavelength UV light. Detection sensitivity was greatly increased, and characteristic color shifts were observed by spraying with the ethanolamine reagent (NH_2EtOH, see the Experimental Section). Under these conditions, the avenanthramides appeared bright yellow in daylight and varied from dull brown, green, and blue to fluorescent yellow, yellow-green, green, blue, orange, red, and white in UV. Although the silica gel TLC systems clearly resolved most of the avenanthramides, preparative-scale separations could not be used due to breakdown of the compounds on the layer during drying. Similar degradation also occurred during solvent evaporation when silica gel column fractionation was attempted. Therefore, all preparative-scale purification was carried out by repeated chromatography on Sephadex LH-20 columns using the TLC systems to monitor purity at each step. The chromatographic properties and detection characteristics are summarized in Table I.

Structural Elucidation. Although the groat and hull extracts differed in their complements of avenanthramides, some 15 were common to both tissues and, of these, 10 major components deleted in both hull and groat extracts, have so far been purified in sufficient quantities for structural analyses. Avenanthramide A (1) crystallized from hot aqueous acetone as pale yellow needles, mp 277 °C. EIMS indicated a molecular ion radical $[M]^{\bullet+}$ at m/z 299 (36%) corresponding to a molecular composition $C_{16}H_{13}NO_5$ (HRMS: 299.0796 observed; 299.0794 calculated). The base peak at m/z 147 (100%) was accompanied by a series of peaks at m/z 119 (20%), 91 (18%), and 65 (11%), correlating with the successive loss of two CO (28 mu) and one C_2H_2 (26 mu) fragments from a monohydroxylated cinnamoyl moiety. Another series at m/z 135 (15%), 107 (4%), and 80 (2%) corresponded to the loss

Table I. Chromatographic Properties of Oat Avenanthramides

avenanthramide		TLC color		mean chromatogr mobility K' (range) on Sephadex LH-20 in various solvent systems[a]				
compd	no.	UV	UV + NH_2EtOH	I	II	III	IV	V
A	1	dull brown	dull green	>10	>10	5.32 (4.94–5.70)	3.53 (3.13–3.92)	9.1 (8.6–9.6)
A-1	2	dull brown	dull green	>10	>10	4.56 (4.17–4.94)	2.77 (3.41–3.13)	8.1 (7.6–8.6)
B	3	dull brown	fl yellow-green	>10	5.64 (5.09–6.18)	3.04 (2.78–3.29)	2.04 (1.82–2.26)	7.10 (6.30–7.90)
B-1	4	dull brown	fl yellow-green	>10	4.74 (4.38–5.09)	2.55 (2.32–2.78)	1.72 (1.62–1.82)	5.94 (5.57–6.30)
C	5	light gray	fl blue	>10	>10	8.79 (8.17–9.4)	4.67 (4.33–5.00)	>10
C-1	6	light gray	fl blue	>10	>10	7.70 (7.23–8.17)	4.07 (3.81–4.33)	9.27 (8.00–10.5)
D	7	fl blue	fl blue-green	7.01 (6.61-7.40)	3.16 (2.82-3.51)	2.33 (2.06-2.59)	<1.0	8.33 (7.14-9.52)
D-1	8	fl blue	fl blue-green	6.15 (5.69-6.61)	2.52 (2.22-2.82)	1.85 (1.63-2.06)	<1.0	6.43 (5.71-7.14)
E	9	dull yellow	fl green	2.94 (2.63-3.25)	2.10 (1.88-2.31)	1.39 (1.26-1.52)	<1.0	6.41 (5.42-7.39)
E-1	10	dull yellow	fl green	2.30 (2.06-2.53)	1.65 (1.42-1.88)	1.16 (1.06-1.26)	<1.0	4.66 (4.14-5.19)

[a] Solvent $CHCl_3$–C_6H_{12}–MeOH–HOAc: I, 50:40:5:5; II, 50:35:10:5; III, 50:30:15:5; IV, 50:25:20:5. Solvent CH_3COCH_3–H_2O–HOAc: V, 30:65:5.

of H_2O (18 mu), CO (28 mu), and HCN (27 mu) from the ion radical $[C_7H_7NO_3]^{\bullet+}$ at m/z 153 (23%).

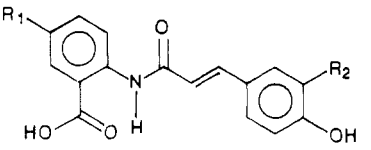
The ^1H NMR spectrum of avenanthramide A in acetone-d_6 showed the presence of seven aromatic protons and a two-proton AB spin-coupled system (δ 6.56, 7.55; J_{AB} = 15.5 Hz) attributable to an *E*-oriented olefinic function. Spin decoupling of the aromatic protons estabilished the substitution patterns of protons on two separate rings. One aromatic ring contained a four-proton AA'BB' system (δ 6.85 (d), 7.52 (d); $J_{AB} = J_{A'B'}$ = 8.5Hz; 1,4-disubstitution), and the other, a three-proton ABX system (δ 8.58 (d), 7.49 (d), 7.07 (dd); J_{AB} = 9.2 Hz, J_{BX} = 3.0 Hz, J_{AX} = 0.3 Hz; 1,2,4- or 1,3,4-trisubstitution).

When subjected to alkaline hydrolysis (2 N NaOH, 100 °C, 8 h), avenanthramide A gave two acidic phenolics, recovered by anion exchange. One was readily identified as 4-hydroxy-(*E*,*Z*)-cinnamic acid (*p*-coumaric acid) by cochromatography with an authentic standard. The other exhibited a brilliant sky blue fluoresence in UV light both in solution and on TLC plates but underwent gradual decomposition in air and daylight to a mixture of purple, green, and orange-red degradation products. It gave a positive test with acidified 4-(dimethylamino)benzaldehyde (yellow in daylight, bright orange fluorescence in UV), indicating the presence of an aromatic primary amine function. EIMS revealed a molecular ion radical $[M]^{\bullet+}$ at m/z 153 (100%) $[C_7H_7NO_3]$ with major fragments at m/z 135 (90%), 107 (75%), and 80 (18%) as seen in the MS of avenanthramide A and allowed postulation as hydroxylated aminobenzoic acid. Comparative chromatography and UV and MS studies showed the acid to be identical with 5-hydroxyanthranilic acid synthesized from 5-chloro-2-nitrobenzoic acid (see the Experimental Section). The above spectral and hydrolytic evidence suggested avenanthramide A to be *N*-(4'-hydroxy-(*E*)-cinnamoyl)-5-hdyroxyanthranilic acid. This postulation was confirmed by total synthesis.

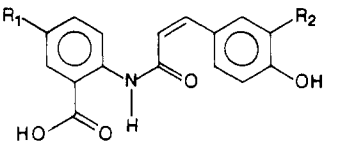
Avenanthramide A-1 (**2**) exhibited similar alkali color shifts and MS properties to avenanthramide A but was chromatographically distinct. In fact, 2-dimensional TLC using the same solvent (solvent B), with brief exposures to daylight or UV light between developments, showed that avenanthramides A and A-1 were interconvertible. If kept in darkness between runs, the two avenanthramides remained distinct. ^1H NMR studies were therefore carried out on avenanthramide A in acetone-d_6 before and after in situ UV irradiation of the filled NMR tube following the procedures of Wagner and Burghart (1981). Irradiation caused a marked decrease in the intensity of the olefinic doublets of the hydroxycinnamoyl moiety at δ 6.56 and 7.55 (J_{AB} = 15.5 Hz) and the concomitant appearance of two new doublets at δ 5.97 and 6.80 (J'_{AB} = 12.5 H) attributable to the same olefinic protons but now in the *Z* configuration. Minor "shifts" were also observed for most of the other protons, most notably the 3',5' doublet ($\Delta\delta$ −0.12), the 2',6' doublet ($\Delta\delta$ +0.09), and the H-6 doublet ($\Delta\delta$ +0.06). These results indicate that avenanthramide A and A-1 are the respective *E* and *Z* isomers of the same compound. Furthermore, unless precautions are taken to exclude UV or daylight during extraction and workup, both forms are likely to be encountered. Similar hydrolytic, NMR, and MS analyses of avenanthramide B (**3**) ($C_{17}H_{15}NO_6$) and C (**5**) ($C_{16}H_{13}NO_6$) estalished their structures as *N*-(4'-hydroxy-3'-methoxy-(*E*)-cinnamoyl)- and *N*-(3',4'-dihydroxy-(*E*)-cinnamoyl)-5-hydroxyanthranilic acid, respectively. The structures of avenanthramides B-1 (**4**) and C-1 (**6**) as the *Z* isomers of B and C, respectively, followed from ^1H NMR photoisomerization studies as described above. Avenanthramide pairs D (**7**) ($C_{16}H_{13}NO_4$), D-1 (**8**) and E (**9**) ($C_{17}H_{15}NO_5$), E-1 (**10**) were similarly formulated as the corresponding *E* and *Z* isomer pairs for *N*-(4'-hydroxycinnamoyl)- and *N*-(4'-hydroxy-3'-methoxycinnamoyl)anthranilic acid, respectively. These assignments for avenanthramides D and E were confirmed by total synthesis. The structures for the 10 avenanthramides including their *E* or *Z* isomeric form are shown in Table II. Table III outlines the major diagnostic ions in the MS fragmentation of avenanthramides while ^1H NMR and ^{13}C NMR parameters are summarized in Tables IV and V, respectively.

Spectral Properties. To identify potentially useful diagnostic correlations between spectral characteristics and structural/substitution patterns, a cursory study was made of avenanthramide UV absorption spectra. Using recrystallized *E* forms, spectra were recorded between 210 and 500 nm in 100% methanol both before and after the addition of aqueous NaOH. Absorption maxima and inflection points (superscript s) for avenanthramides A–E are summarized in Table VI. The effects of alkali-induced ionization of carboxylic and phenolic hydroxyl groups on absorption maxima are included. In general, the avenanthramides exhibit principal absorption maxima (Band I) in the range 325–355 nm and appear pale yellow to yellow-green in solution. This band I maximum represents in part the electron-transfer (ET) transitions typical of hydroxycinnamoyl functions (e.g., band I range 308–330 nm; ϵ approximately 10^4–10^5; Harborne, 1964; Scott, 1964). Its appearance at somewhat longer wavelengths (10–25 nm) is probably due to the bathochromic effect of extended double-bond conjugation through the *o*-amidobenzoic acid moiety. The anticipated two-band pattern for the ET bands of the *o*-amidobenzoyl chromophore (Scott, 1964) is not evident in any of the avenanthramide spectra. However, a number of inflection points appear on the

Table II. Structures of Oat Avenanthramide Alkaloids



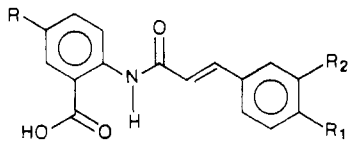
E form



Z form

chromatogr designation	no.	olefinic confign	R_1	R_2	structure
A	1	*E*	OH	H	*N*-(4′-hydroxycinnamoyl)-5-hydroxyanthranilic acid
A-1	2	*Z*			
B	3	*E*	OH	OCH_3	*N*-(4′-hydroxy-3′-methoxycinnamoyl)-5-hydroxyanthranilic acid
B-1	4	*Z*			
C	5	*E*	OH	OH	*N*-(3′,4′-dihydroxycinnamoyl)-5-hydroxyanthranilic acid
C-1	6	*Z*			
D	7	*E*	H	H	*N*-(4′-hydroxycinnamoyl)anthranilic acid
D-1	8	*Z*			
E	9	*E*	H	OCH_3	*N*-(4′-hydroxy-3′-methoxycinnamoyl)anthranilic acid
E-1	10	*Z*			

Table III. Principal Ions in the Mass Spectra of Oat Avenanthramides



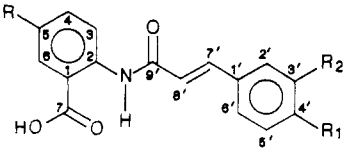
	A	B	C	D	E
R	OH	OH	OH	H	H
R_1	OH	OH	OH	OH	OH
R_2	H	OCH_3	OH	H	OCH_3
MW	299	329	315	283	313
	Major Ions (% Abundance)				
$M^{\cdot+}$	299 (36)	329 (23)	315 (20)	283 (20)	313 (58)
$[M - H_2O]^{\cdot+}$	281 (7)	311 (3)	297 (30)	265 (2)	295 (3)
$[a + H]^{\cdot+}$	153 (22)	153 (11)	153 (65)	135 (11)	135 (5)
$[a - OH]^{\cdot+}$	135 (12)	135 (5)	135 (40)	117 (12)	117 (5)
$[C]^+$	147 (100)	177 (100)	163 (100)	147 (100)	177 (100)
$[C - CO]^+$	119 (20)	149 (4)	135 (40)	119 (24)	149 (10)

short-wavelength side of band I (i.e., band II region). From spectral studies using model hydroxycinnamoyl esters and *N*-acylanthranilates, these fine-structure shoulders are not attributable to either the hydroxycinnamoyl or amidobenzoyl moieties when examined separately and may therefore be characteristic of the cross-conjugated cinnamoylamidobenzoate chromophore to avenanthramides. The absorption characteristics of the avenanthramides below 260 nm are more complex, arising from the superposition of band II from the cinnamoyl (Harborne, 1964) and band III from the *o*-amidobenzoyl (Scott, 1964) chromophores.

Comparison among the avenanthramides indicates that hydroxylation of the amidobenzoic acid moiety meta to the carboxyl function (i.e., para to the amido function) produces a bathochromic shift in band I of about 12–15 nm (A vs D; B vs E). Subsitution of the cinnamoyl function at the 3′-position produces less of a shift (D vs E; 5 nm; A vs B; 9 nm). These substitutions have little effect on the amidobenzoic acid chromophore.

Ionization of the carboxylic and phenolic hydroxyl groups with strong base produces considerable bathochromic shifts in band I in all avenanthramides ($\Delta\lambda_{max} \approx$ +35 to +50 nm) but has little effect on the band II region,

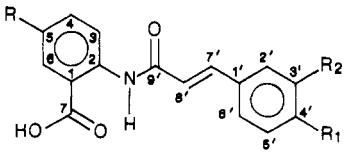
Table IV. ^{1}H NMR Parameters[a] of Oat Avenanthramides



	A	B	C	D	E
R	OH	OH	OH	H	H
R_1	OH	OH	OH	OH	OH
R_2	H	OCH_3	OH	H	OCH_3
H-2′	7.52 (d, $J_{2',3'}$ = 8.5)	7.29 (d, $J_{2',6'}$ = 1.8)	7.05 (d, $J_{2',6'}$ = 2.0)	7.53 (dt, $J_{2',3'}$ = 8.6, $J_{2',6'}$ = 1.4)	7.33 (d, $J_{2',6'}$ = 1.9)
H-6′		7.10 (dd, $J_{6',2'}$ = 1.8, $J_{6',5'}$ = 8.1)	6.96 (dd, $J_{6,2}$ = 2.0, $J_{6,5}$ = 8.1)		7.13 (dd, $J_{6',2'}$ = 1.9, $J_{6',5'}$ = 8.1)
H-3′	6.85 (d, $J_{3',2'}$ = 8.5)			6.82 (dt, $J_{3',2'}$ = 8.6, $J_{3',5'}$ = 1.4)	
H-5′		6.81 (d, $J_{5',6'}$ = 8.1)	6.75 (d, $J_{5',6'}$ = 8.1)		6.81 (d, $J_{5',6'}$ = 8.1)
H-7′	7.55 (d, $J_{7',8'}$ = 15.5)	7.50 (d, $J_{7',8'}$ = 15.5)	7.37 (d, $J_{7',8'}$ = 15.5)	7.57 (d, $J_{7',8'}$ = 15.5)	7.53 (d, $J_{7',8'}$ = 15.5)
H-8′	6.56 (d, $J_{8',7'}$ = 15.5)	6.67 (d, $J_{8',7'}$ = 15.5)	6.46 (d, $J_{8',7'}$ = 15.5)	6.61 (d, $J_{8',7'}$ = 15.5)	6.70 (d, $J_{8',7'}$ = 15.5)
H-3	8.58 (d, $J_{3,4}$ = 9.2)	8.40 (d, $J_{3,4}$ = 9.0)	8.31 (d, $J_{3,4}$ = 9.0)	8.67 (dd, $J_{3,4}$ = 8.5, $J_{3,5}$ = 0.8)	8.63 (dd, $J_{3,4}$ = 8.0, $J_{3,5}$ = 0.8)
H-4	7.07 (dd, $J_{4,3}$ = 9.2, $J_{4,6}$ = 3.0)	7.04 (dd, $J_{4,3}$ = 9.0, $J_{4,6}$ = 3.0)	6.99 (dd, $J_{4,3}$ = 9.0, $J_{4,6}$ = 3.0)	7.56 (ddd, $J_{4,3}$ = 8.5, $J_{4,5}$ = 7.7, $J_{5,3}$ = 1.7)	7.60 (ddd, $J_{4,3}$ = 8.0, $J_{4,5}$ = 7.9, $J_{4,6}$ = 1.6)
H-5				7.11 (ddd, $J_{5,6}$ = 7.9, $J_{5,4}$ = 7.7, $J_{5,3}$ = 0.8)	7.15 (ddd, $J_{5,6}$ = 7.9, $J_{5,4}$ = 7.9, $J_{5,3}$ = 0.8)
H-6	7.49 (d, $J_{6,4}$ = 3.0)	7.41 (d, $J_{6,4}$ = 3.0)	7.36 (d, $J_{6,4}$ = 3.0)	8.01 (dd, $J_{6,5}$ = 7.9, $J_{6,4}$ = 1.7)	8.00 (dd, $J_{6,5}$ = 7.9, $J_{6,4}$ = 1.6)

[a] Chemical shifts in δ; coupling constants in hertz. Avenanthramide A in acetone-d_6; all others in DMSO-d_6.

Table V. ^{13}C NMR Parameters[a] of Oat Avenanthramides



	A	B	D	E
R	OH	OH	H	H
R_1	OH	OH	OH	OH
R_2	H	OCH_3	H	OCH_3
C-1	118.5	118.6	116.6	116.6
C-2	133.1	133.3	141.5	141.3
C-3	122.5	122.8	120.4	120.4
C-4	121.0	121.0	134.2	134.1
C-5	152.6	152.8	122.7	122.9[b]
C-6	116.6	116.8	131.4	131.2
C-7	163.9	164.1	164.5	164.5
C-1′	125.7	126.4	125.7	126.1
C-2′	129.9	111.5	130.2	111.5
C-3′	115.8	149.1	116.0	149.1
C-4′	159.4	148.1	159.8	148.1
C-5′	115.8	115.9	116.0	115.8
C-6′	129.9	122.8	130.2	122.7[b]
C-7′	140.8	141.4	141.8	142.1
C-8′	119.1	119.5	118.9	119.0
C-9′	169.3	169.5	169.9	169.7
OCH_3		55.9		55.8

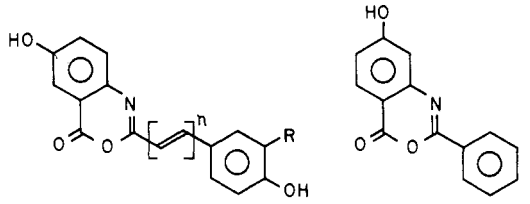
[a] Chemical shifts in δ; all spectra in DMSO-d_6. [b] Assignments interchangeable.

which becomes more clearly discernible in alkali. Such shifts can be attributed primarily to the ionized hydroxycinnamoyl chromophore rather than the amidobenzoic acid moiety since both *N*-acetylanthranilic and *N*-acetyl-5-hydroxyanthranilic acid model compounds showed little change under these condition. Ethyl ferulate on the other had showed an alkali shift of +50 nm. As might be anticipated, the ortho-dihydroxylated avenanthramide C rapidly decomposes in strong alkali.

Physicochemical and Biological Properties. The avenanthramides are high melting point pale yellow to yellow-green crystalline substances. They are freely soluble in ethyl acetate, diethyl ether, and aqueous mixtures with acetone or the lower alcohols but are relatively insoluble in chloroform, benzene, or water. At alkaline pH they readily dissolve even in cold water, giving bright yellow to green solutions. As a group, the avenanthramides show high affinity for binding to nylons, polymides, and presumably other "peptide" (–NHCO–) polymers. They are resistant to acid hydrolysis but are slowly hydrolyzed with some decomposition in alkali to the corresponding substituted cinnamic and anthranilic acids. The hydroxyanthranilic acids (i.e., for A–C) are extremely sensitive to photoinduced and O_2-induced autooxidation, resulting in the progressive formation of orange–red–brown–black condensed resins.

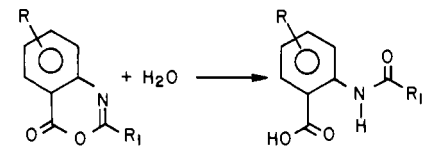
As shown by multiple-development TLC with and without prior exposure to UV light, both *E* and *Z* isomers exist in solution as a photomediated interconvertable mixture. At equilibrium, the *E* isomer predominates, and the photoconversion resembles that observed with other hydroxycinnamate derivatives (Challice and Williams, 1966; Hartley and Jones, 1975). Determination of whether the *E* isomer only or both isomers are naturally occurring in oat tissues would require that extraction, purification, and estimatation be carried out in the absence of UV and daylight. Because of a much lower solubility, crystallization from aqueous acetone produces the pure *E* isomer.

The avenanthramides are closely related to the avenalumins **11** (R = H, OCH_3; n = 1, 2), recently described phytoalexin alkaloids produced in oat leaves following infective germination of crown-rust spores *Puccinia coronata* f.sp. *avenae*. The avenalumins (2-styryl-1,3-benz-



11 12

oxazin-4*H*-ones) displayed potent antifungal activity in vitro and appear to be quantitatively associated with specific resistance expression in the interactions between oat cultivars and rust races (Mayama, 1983). Similarly, dianthalexin (**12**), a 2-phenyl-1,3-benzoxazin-4*H*-one analogue, has also been reported as an antifungal component produced in carnations in response to elicitation and infection by *Phytophthora parasitica* (Bouillant et al., 1983). However, 2-aryl-1,3-benzoxazin-4*H*-ones readily undergo hydrolysis in aqueous media to give the corresponding (*N*-aroylamido)benzoic acids (Williams and Salvadori, 1971; Errede et al., 1980) as shown below:



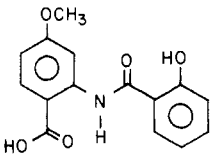
Considering the instability of these 2-aryl-1,3-benzoxazin-4*H*-ones in water, it might be anticipated that the *N*-aroylanthranilate rather than the 2-aryl-1,3-benzoxazin-4*H*-one form is the biologically active phytoalexin. In support of the conclusion, Ponchet et al. (1984) have reinvestigated the carnation phytoalexins and reported the occurrence of two additional antifungal agents, the di-

Table VI. UV Spectral Properties of Oat Avenanthramides

avenanthramide		major absorption bands III	II	I	I
A	MeOH	220	298,[s] 305[s]	322, 339	
	MeOH + NaOH	214, 233[s]	308,[s] 317[s]	379	+40 nm
B	MeOH	225, 242	304[s]	334,[s] 354	
	MeOH + NaOH	228, 254[s]	306,[s] 315	361, 392	+38 nm
C	MeOH	220,[s] 240[s]	303[s]	330,[s] 345	
	MeOH + NaOH	214, 236,[s] 266[s]	302,[s] 314[s]	346,[s] 388[s]	+43 nm
D	MeOH	218	294,[s] 302[s]	329	
	MeOH + NaOH	213, 233[s]	306,[s] 314	371	+42 nm
E	MeOH	211, 236,[s] 246[s]	292,[s] 304[s]	338	
	MeOH + NaOH	219, 262[s]	301, 310[s]	385	+47 nm

[a] Compound unstable in alkali.

anthramides A and B (**13**, **14**), from elicited tissues. It is



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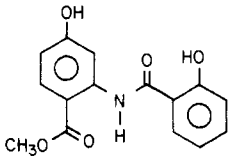

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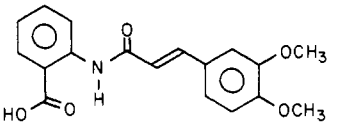
not known whether the dianthramides are artifacts of hydrolyzed benzoxazinones or whether they occur as such in the tissues before extraction. In this study, because aqueous ion-exchange techniques were used to fractionate the oat extracts, the presence/absence of avenalumins in the oat tissues before extraction could not be confirmed. Experiments using nonaqueous extraction solvents and/or rapid identification procedures will be necessary to establish the presence of avenalumins in the grain.

An extensive literature review indicates that the avenanthramides described in this paper are novel natural products. Several *N*-cinnamoylanthranilic acids however have been previously described in pharmaceutical patents and are known to possess antiallergic, antihistaminic, and antiasthmatic activity (for review see Devlin and Hargrave (1985)). In fact, original patent claims of therapeutic activity include *N*-(4′-hydroxycinnamoyl)anthranilic (i.e., avenanthramides D, D-1), *N*-(4′-hydroxy-3′-methoxycinnamoyl)anthranilic (i.e., avenanthramides E, E-1), and *N*-(3′,4′-dimethoxycinnamoyl)anthranilic acids (i.e., monomethyl ethers of avenanthramides E, E-1). This latter compound is marketed under the trade names of Tranilast and Rizaben (**15**) and, interestingly, although no mention



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was made in the original Japanese patents as to possible isomeric differences, the *Z* isomer has been recently shown to possess over 10 times the antiallergic activity of the *E* isomer (Kakegawa et al., 1985). The 4′-desmethyl compound (i.e., avenanthramides E, E-1) has just been patented as an in vitro lipoxygenase inhibitor (Wakabayashi et al., 1986).

The two-dimensional TLC system developed for oat extracts revealed over 40 different avenanthramide-like compounds in hull and groat tissues with provisional identification as avenanthramides based on chromatographic, diagnostic color responses and preliminary MS data. This diversity of closely related phenolics has made isolation and identification difficult. Furthermore, the quantification of avenanthramides is complicated by different absorption spectra for each derivative. Attempts are currently under way to develop an HPLC technique to allow rapid quantitative separation and estimation of the avenanthramides and to address the problem of *E* to *Z* isomerization.

ACKNOWLEDGMENT

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Registry No. 1, 108605-70-5; **2**, 116764-20-6; **3**, 108605-69-2; **4**, 116764-21-7; **5**, 116764-15-9; **6**, 116764-22-8; **7**, 115610-36-1; **8**, 116764-23-9; **9**, 93755-77-2; **10**, 116764-24-0; 5-chloro-2-nitrobenzoic acid, 2516-95-2; 5-hydroxy-2-nitrobenzoic acid, 610-37-7; 5-hydroxyanthranilic acid, 394-31-0; 4-acetoxy-(*E*,*Z*)-cinnamoyl chloride, 53901-95-4; 4-hydroxy-(*E*,*Z*)-cinnamic acid, 7400-08-0; 4-acetoxy-(*E*,*Z*)-cinnamic acid, 15486-19-8; 4-acetoxy-3-methoxy-(*E*,*Z*)-cinnamoyl chloride, 50906-12-2; 4-hydroxy-3-methoxy-(*E*,*Z*)-cinnamic acid, 1135-24-6; anthranilic acid, 118-92-3; 4′-acetoxy-3′-methoxy-(*E*,*Z*)-cinnamoyl chloride, 50906-12-2.

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