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A STRESS COMPOUND IN OATS INDUCED BY VICTORIN, A HOST-SPECIFIC TOXIN FROM *HELMINTHOSPORIUM VICTORIAE*

HISASHI MIYAGAWA,* ATSUSHI ISHIHARA, YASUMASA KUWAHARA, TAMIO UENO† and SHIGEYUKI MAYAMA‡

Pesticide Research Institute, Faculty of Agriculture, Kyoto University, Kyoto 606-01, Japan; †Department of Agricultural Chemistry, Kyoto University, Kyoto 606-01, Japan; ‡Faculty of Agriculture, Kobe University, Kobe 657, Japan

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Key Word Index—*Avena sativa*; Gramineae; oats; anthranilic acid derivative; avenanthramide G; stress compound; victorin.

Abstract—A stress compound in oats characteristically produced upon treatment with victorin, a host-specific toxin from *Helminthosporium victoriae*, was isolated. Its structure was shown to be *N*-(4′-hydroxycinnamoyl)-4-hydroxyanthranilic acid (avenanthramide G), by spectroscopic analyses and chemical synthesis.

INTRODUCTION

Oat leaves, when inoculated with an incompatible race of crown rust fungus, produce phytoalexins, associated in a significant manner with the resistance to the pathogen [1, 2]. The structures of the oat phytoalexins have been shown to be a series of substituted *N*-cinnamoyl anthranilic acid derivatives, commonly referred to as avenanthramides (**2**–**4**, formerly called avenalumins) [3]. It has been also demonstrated that victorin, a host-specific toxin from *Helminthosporium victoriae*, is a potent elicitor of the phytoalexins in oat cultivars which carry the *Pc*-2 gene [4]. During our studies of the elicitor activity of victorin, we have found that, in addition to avenanthramide A, a major oat phytoalexin, victorin induces another metabolite barely detectable in the interaction between oats and the rust fungus. The present report deals with the isolation and structural characterization of this metabolite, which is induced by victorin.

RESULTS AND DISCUSSION

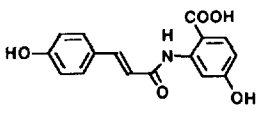
After treating oat leaves (cv. Pc-2) with victorin D for 24 hr (nomenclature according to Mayama *et al.* [4]) at a concentration of 1 ng ml^{-1}, the induction of the metabolite (**1**) was observed by reversed phase HPLC (column; Wakosil II C18, 4.6 × 150 mm, mobile phase; 50% MeOH in water containing. 0.1% H_3PO_3, 0.8 ml min^{-1}, detection; UV340 nm) having a R_t 8.7 min, along with avenanthramide A (R_t 6.4 min). In order to prepare **1** in larger amounts, we searched for an alternative elicitor to victorin, since it is available in only limited quantities, and silver nitrate ($AgNO_3$) was found to be effective for the induction of **1**.

Seven-day-old oat seedlings (cv. Shokan 1) were sprayed with aqueous $AgNO_3$ (1000 ppm). After two days, the aerial portion (700 g) was extracted with MeOH, and the extract was purified by solvent partitioning, ODS column chromatography and reversed phase HPLC to afford pure **1** (15.7 mg).

Thermospray LCMS analysis of **1** as well as EI-Mass Spectrometry analysis after acetylation gave spectra nearly identical with those of avenanthramide A (**2**), suggesting that **1** might be an isomer of **2**. A comparison of the ^{1}H and ^{13}C NMR spectra of **1** (δ_H: 6.53, 6.81 (2H), 7.49 (2H), 7.59; δ_C: 115.9, 119.0, 125.6, 130.1, 141.1, 159.6) with those of **2** suggested the presence of 4-hydroxycinnamoyl moiety in **1**, which was supported by hydrolysis of **1**; 4-hydroxycinnamic acid was identified in the alkaline hydrolysate of **1** by comparing its HPLC chromatographic behaviour and UV spectrum with those of an authentic sample. Moreover, the ^{1}H NMR spectrum indicated that **1** also contains a 1,2,4-trisubstituted phenyl moiety (δ_H: 6.50, 7.95, 8.19). Based on the assumption that **1** is an isomer of **2**, these signals were assigned to a 4-hydroxyanthranilic acid moiety. Therefore the structure of **1** is *N*-(4′-hydroxycinnamoyl)-4-hydroxyanthranilic acid.

This was confirmed by synthesis (scheme 1). 4-Hydroxyanthranilic acid (**8**) was prepared from 2,4-dinitrobenzoic acid (**5**) in 3 steps according to Reinhard and Matern [5] and Drain *et al.* [6] and condensed with 4-acetoxycinnamoyl chloride (**9**), prepared from 4-hydroxycinnamic acid by acetylation and conversion to the acid chloride. The resulting amide (**10**) was then deprotected under mild alkaline conditions to afford **1**. The physicochemical data (^{1}H and ^{13}C NMR, IR, UV and

*Present address: Department of Agricultural Chemistry, Kyoto University, Kyoto 606-01, Japan.



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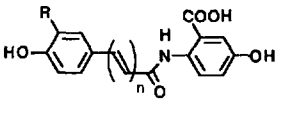


2: R = H, n = 1
3: R = CH_3O, n = 1
4: R = H, n = 2

mass spectrum) of synthetic **1** were identical with those of the natural compound.

Compound **1** has been described as a component of the hulls and bran of oats, and has been named avenanthramide G [7], although its isolation and data relative to its chemical constitution have not been presented. It is induced, along with **2**, as a result of stress caused by the toxic effect of victorin or $AgNO_3$. Neither **1** nor **2** play a defensive role against *H. victoriae*, the producer of victorin, because they show no substantial inhibitory activity against the spore germination of the fungus *in vitro* (data not shown).

In oats, it has been established that the phytoalexins **2**–**4** accumulate in association with the incompatibilities between oat cultivars and rust fungus races [1, 2]. Compound **1** is structurally related to these phytoalexins, although the phytoalexins commonly contain 5-hydroxyanthranilic acid moieties. An *N*-acyl 4-hydroxyanthranilic acid has also been isolated from groats and hulls of oats, named AF-8 [8].

EXPERIMENTAL

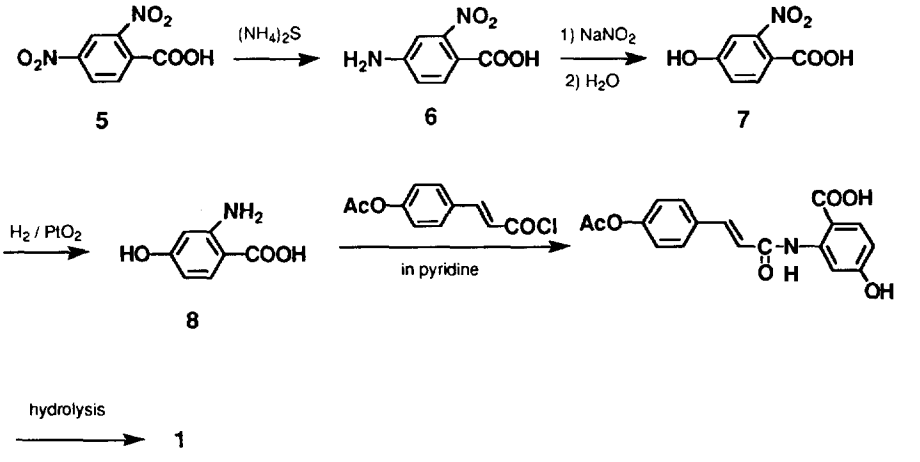
Plant material. Oats (*Avena sativa* L. cv. Shokan 1) were grown in vermiculite at 25° in a growth chamber with a 12 hr period of illumination with fluorescent lamps. For the elicitation, 7 day old seedlings were sprayed with aq. solution of $AgNO_3$ (1 mg ml^{-1}).

Extraction and isolation of **1**. The aerial portions of oats (700 g) were extracted with MeOH 2 days after treatment. After filtration, the solvent was evapd to dryness under reduced pressure. The residue was then dissolved in MeOH–H_2O (4:1) and washed with *n*-hexane. The aq layer was diluted 4-fold with 1% aq. HOAc and applied to an ODS column (Cosmosil 75C18-OPN, 4 × 25 cm). The column was first washed with MeOH–H_2O–HoAc (200:798:2) and then eluted with MeOH–H_2O–HoAc (100:99:1). The eluate was concd and further purified by reversed phase HPLC (column: Cosmosil 5C18 AR, 20 × 250 mm, solvent: MeOH–H_2O–HOAc (275:223:2), 6 ml min^{-1}, detection: UV 280 nm) to afford **1** (15.7 mg) as a yellow amorphous powder. Physicochemical data for **1**: UV λ_{max} (MeOH) nm: 326, 259, 217. IR ν_{max} (KBr) cm^{-1}: 3320, 1600, 1225. Thermospray-LCMS *m/z*: 300 ($M + H^+$), 282, 256. ^{1}H NMR (CD_3OD) δ: 6.50 (1H, *d*, J = 15.8 Hz), 6.53 (1H, *dd*, J = 9.0, 2.5 Hz), 6.81 (2H, *d*, J = 8.8 Hz), 7.49 (2H, *d*, J = 8.8 Hz), 7.59 (1H, *d*, J = 15.8 Hz), 7.97 (1H, *d*, J = 9.0 Hz), 8.19 (1H, *d*, J = 2.5 Hz). ^{13}C NMR (DMSO-d_6) δ: 105.7, 108.7, 109.6, 115.6, 118.9, 125.3, 129.7, 133.2, 140.9, 143.0, 159.2, 161.7, 163.9, 169.5.

Acetate of **1**. EI-MS *m/z*: 365 (M^+), 323, 281, 280, 253, 252, 236, 147, 135, 119, 106, 91, 43. ^{1}H NMR (CD_3OD) δ: 2.32 (3H, *s*), 2.37 (3H, *s*), 6.72 (1H, *d*, J = 16.0 Hz), 7.16 (2H, *d*, J = 8.3 Hz), 7.26–7.38 (2H, *m*), 7.61 (1H, *d*, J = 8.3 Hz), 7.86 (1H, *d*, J = 16.0 Hz), 8.23 (1H, *d*, J = 8.6 Hz).

Alkaline hydrolysis of **1**. Purified **1** (1 mg) was dissolved in 2N NaOH (0.5 ml) and refluxed for 12 hr. The reaction mixture was then neutralized with 1N HCl followed by filtration. The filtrate was then desalted using a Sep-Pak C18 cartridge. The desalted hydrolysate was analysed by gradient HPLC (column, Wakosil-II 5C18 HG 4.6 × 150 mm; solvent, starting with 20% MeOH in water containing 0.1% H_3PO_3 for 5 min, then linearly increasing the concn of MeOH to 80% in 25 min; flow rate, 0.8 ml min^{-1}; detection, UV 280 nm). The column effluent corresponding to the appropriate peak was collected, and the UV spectrum was measured in order to identify the material. 4-Hydroxycinnamic acid was detected at R_t 16.2 min, with UV λ_{max} in MeOH at 312 and 300 (sh) nm.

Synthesis. 4-*Hydroxyanthranilic acid* (**8**). According to Reinhard and Matern [5], 2,4-dinitrobenzoic acid (**5**, 5 g)



Scheme 1.

was reduced to 2-nitro-4-aminobenzoic acid (**6**) with ammonium sulphide (15% aqueous solution). Compound **6** was extracted from the reaction mixture as described in the literature, and then transferred under mildly acidic conditions (pH 5) to MeOAc for desalting. Recrystallization from H_2O–HOAc gave **6** in 57% yield as dark brown powdery crystals, mp 236.5–239.5°. EI-MS *m/z*: 182 (M^+), 165, 136, 108, 80. ^1H NMR (CD_3OD) δ: 6.75 (1H, *d*, $J = 9.3$ Hz), 6.76 (1H, s), 7.66 (1H, *d*, $J = 9.3$ Hz). Subsequently, **6** (1.82 g) was converted to 4-hydroxy-2-nitrobenzoic acid (**7**) via the diazonium salt, as described by Drain *et al.* [6]. Purification by silica gel using $CHCl_3$–MeOH (1:1) afforded **7** as brown crystals (558 mg, 31.6%), mp 230–232°. EIMS *m/z*: 183 (M^+), 139, 137, 109, 81. ^1H NMR (CD_3OD) δ: 7.03 (1H, *d*, $J = 8.5$ Hz), 7.08 (1H, *s*), 7.77 (1H, *d*, $J = 8.5$ Hz). Finally, the nitro group of **7** was reduced by catalytic hydrogenation, using a soln of **6** (558 mg) in EtOH (12 ml) with PtO_2 (20 mg). The soln was then stirred under H_2 for 3 hr. After filtration, followed by solvent evapn, the residue was crystallized from H_2O, and the resulting crystals were purified by HPLC (column, Cosmosil 5C18 AR 20 × 250 mm; solvent, MeOH–H_2O–HOAc (100:981:9); flow rate, 6 ml min^{-1}; detection, UV 280 nm) to give **8** as needles (271 mg, 58.2%), mp 154° (decomp.). The measurement was hampered by the sample sublimation, and the observed mp value varied with the heating rate. Analysis found: C, 54.71; H, 4.47; N, 9.04%. Calc. for $C_7H_7O_3N$: C, 54.90; H, 4.46; N, 9.15%. UV λ_{max} MeOH nm (ε): 320 (6,500), 260 (13,000), 225 (43,000). EI-MS *m/z*: 153 (M^+), 135, 108, 107. ^1H NMR (CD_3OD) δ: 6.09 (1H, *d*, $J = 9.2$ Hz), 6.12 (1H, *s*), 7.69 (1H, *d*, $J = 9.2$ Hz).

N-(4'-hydroxycinnamoyl)-4-hydroxyanthranilic acid, Avenanthramide G (**1**). In a manner similar to that described for the synthesis of avenanthramide A by Collins [9], 4-hydroxycinnamic acid was acetylated with Ac_2O and *p*-toluenesulphonic acid, and converted to the acid chloride with $SOCl_2$. Into a suspension of the chloride (prepared from 1.7 g of 4-acetoxycinnamic acid) in dry Me_2CO (12.3 ml), **8** (187.6 mg) in dry pyridine (12.3 ml) was added, and the mixture was refluxed at 100° for 20 min. After cooling to room temp, the solvent was removed by repeated evapn and washing with Me_2CO–H_2O (4:1). The residue was then dissolved in Me_2CO–HOAc–H_2O (8:1:1) and allowed to stand overnight. After removal of the solvent, the concentrate was refluxed in MeOH–H_2O–conc NH_4OH (5:4:1) to remove the acetyl group, followed by conc under the reduced pressure at 30°. The residual oily material was crystallized with MeOH–1% HOAc in water, and recrystallized from the same solvent to give **1** as crystals, mp 290–291°. Analysis found: C, 63.55; H, 4.53; N, 4.67% Calcd for $C_{16}H_{13}O_5N \cdot 1/4MeOH$: C, 63.51; H, 4.56; N, 4.56%. Thermospray LC-MS *m/z*: 300 ($M + H^+$), 282, 256. EI-MS *m/z*: 299 (M^+), 281, 181, 147, 119, 91. UV λ_{max} (MeOH) nm (ε): 328 (35,000), 304sh (25,000), 264 (16,000), 220 (22,000). IR ν_{max} (KBr) cm^{-1}: 3320, 1600, 1225. ^1H NMR (CD_3OD) δ: 6.51 (1H, *d*, $J = 15.8$ Hz), 6.53 (1H, *dd*, $J = 8.8, 2.5$ Hz), 6.81 (2H, *d*, $J = 8.4$ Hz), 7.49 (2H, *d*, $J = 8.4$ Hz), 7.60 (1H, *d*, $J = 15.8$ Hz), 7.97 (1H, *d*, $J = 8.8$ Hz), 8.21 (1H, *d*, $J = 2.5$ Hz). ^{13}C NMR (DMSO-d_6) δ: 106.2, 107.4, 110.1, 115.9, 119.1, 125.6, 130.1, 133.3, 141.4, 143.5, 159.6, 162.5, 164.3, 169.9.

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