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# Biosynthesis of oat avenanthramide phytoalexins

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**Abstract**

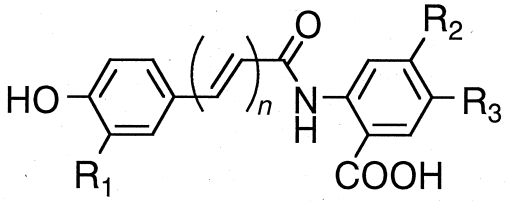
The biosynthesis of oat phytoalexins, avenanthramides, was investigated by administering labeled putative precursors to oat leaf segments treated with the elicitor, penta-*N*-acetylchitopentaose. Among the compounds tested, oat leaf segments incorporated [ring-UL-$^{14}$C]anthranilic acid, [2,3,4,5,6-$^{2}$H]L-phenylalanine and [1,2-$^{13}$C]*p*-coumaric acid into avenanthramides, but not [2,6-$^{2}$H]L-tyrosine. These findings suggested that avenanthramides were de novo synthesized from primary metabolites after elicitor treatment and that phenylpropanoid metabolism is involved in their biosynthesis. In addition to L-phenylalanine and *p*-coumaric acid, sodium [$^{13}C_2$]acetate was incorporated into avenanthramide L, which indicated that the avenalumoyl (5-(4-hydroxyphenyl)-2,4-pentadienoyl) moiety of avenanthramide L was biosynthesized from *p*-coumaric acid by elongation of its side chain with an acetate unit. 

*Keywords: Avena sativa*; Oats; Gramineae; Biosynthesis; Phytoalexin; Avenanthramide; Avenalumic acid

## 1. Introduction

Avenanthramides (**1**–**5**), a series of substituted *N*-cinnamoylanthranilate derivatives, are phytoalexins in oats (*Avena sativa* L.) (Mayama, Tani, & Matsuura, 1981; Mayama, Tani, Iida, & Matsuura, 1982; Miyagawa, Ishihara, Nishimoto, Ueno, & Mayama, 1995; Miyagawa, Ishihara, Kuwahara, Ueno, & Mayama, 1996a). Fungal infection and treatment with various elicitors (Miyagawa, Ishihara, Kuwahara, Ueno, & Mayama, 1996b), such as oligo-*N*-acetylchitooligosaccharides (Bordin, Mayama, & Tani, 1991), victorin C (Mayama, Tani, Ueno, Midland, & Keen, 1986), heavy metal ions (Fink, Liefland, & Mendgen, 1990), and the calcium ionophore A23187 (Ishihara, Miyagawa, Kuwahara, Ueno, & Mayama, 1996), induce their production in oat leaves. Although avenanthramides have also been isolated as constitutive components in oat groats and hulls (Collins, 1989; Collins, McLachlan, & Blackwell, 1991; Collins & Mullin, 1988), none appear to be present in healthy leaves prior to inoculation with pathogens or treatment with elicitors.

The chemical structure of avenanthramides A, B, D and G (**1**–**4**) suggests that these compounds are biosynthesized by condensation of cinnamic acid derivatives and hydroxyanthranilates. Indeed, induction of hydroxycinnamoyl-CoA:hydroxyanthranilate *N*-hydroxycinnamoyltransferase (HHT) activity was recently found in oat leaves after treatment with oligo-*N*-acetylchito-oligosaccharides (Ishihara, Miyagawa, Matsukawa, Ueno, Mayama & Iwamura, 1998) and victorin C (Ishihara et al., 1997).




| | | $R_1$ | $R_2$ | $R_3$ | n |
|---|---|---|---|---|---|
| **1**: Avenanthramide | A | H | H | OH | 1 |
| **2**: | B | OMe | H | OH | 1 |
| **3**: | D | H | H | H | 1 |
| **4**: | G | H | OH | H | 1 |
| **5**: | L | H | H | OH | 2 |

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Table 1
Incorporation of $^{14}C$ into avenanthramides from [ring-UL-$^{14}C$]anthranilic acid

| Avenanthramide | Amount (nmol) | Specific activity (Bq/nmol) | Incorporation (%) | Dilution (fold) |
|---|---|---|---|---|
| A | 121.6 | 99.5 | 15 | 4.7 |
| B | 15.0 | 52.7 | 1.0 | 8.8 |
| D | 3.3 | 96.0 | 0.4 | 4.8 |
| G | 10.8 | 120.3 | 1.6 | 3.9 |
| L | 13.7 | 35.7 | 0.6 | 13.0 |

Compared to these compounds (**1**–**4**), avenanthramide L (**5**) has a unique structure, in which avenalumic acid (5-(4-hydroxyphenyl)-2,4-pentadienoic acid) is linked to 5-hydroxyanthranilic acid, instead of the hydroxycinnamic acids found in **1**–**4**. Since the study of HHT demonstrated that avenalumoyl-CoA serves as a substrate, it is most likely that **5** is biosynthesized by a condensation reaction similar to that for the biosyntheses of **1**–**4**. On the other hand, biosynthesis of the 5-phenyl-2,4-pentadienoic acid structure in avenalumic acid has not been elucidated. Although an analogous structure is found in piperin, a pungent principle in pepper, as piperic acid (5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid), it has only been presumed that its biosynthesis is closely related to that of hydroxycinnamic acids (Collins et al., 1991; Gross, 1981).

This study addresses early steps in the biosynthesis of avenanthramides. By administering labeled, putative precursors to oat leaf segments, it was demonstrated that avenanthramides are de novo synthesized from primary metabolites. Furthermore, the biosynthetic origins of the avenalumoyl moiety in **5** were investigated.

## 2. Results

### 2.1. Administration of [ring-UL-$^{14}C$]anthranilic acid

Oat leaf segments were incubated for 24 h with penta-*N*-acetylchitopentaose in the presence of [ring-UL-$^{14}C$]anthranilic acid. Avenanthramides were then isolated from the elicitor soln by HPLC and the radioactivity of individual avenanthramides were determined (Table 1). Significant amounts of $^{14}C$ were detected in all avenanthramides (**1**–**5**), among which **1** showed the highest radioactivity, amounting to about 15% of the total administered $^{14}C$.

### 2.2. Feeding of [2,3,4,5,6-$^{2}H$]L-phenylalanine and [2,6-$^{2}H$]L-tyrosine

Incorporation of L-phenylalanine into avenanthramides was examined using a stable-isotope labeled compound. After treating oat leaf segments with penta-*N*-acetylchitopentaose in the presence of [2,3,4,5,6-$^{2}$H]L-phenylalanine for 24 h, avenanthramides secreted into the elicitor soln were analyzed by LC-MS. For compounds **1**–**5**, the pseudomolecular ion containing four deuterium atoms (or three atoms in the case of **2**) was clearly observed at $m/z$ $M^+ + 5$ (or $M^+ + 4$) in addition to the pseudomolecular ion of $M^+ + 1$, demonstrating that [2,3,4,5,6-$^{2}$H]L-phenylalanine was incorporated into these compounds with the loss of one deuterium atom (or two atoms). The loss was due to the introduction of a hydroxyl group (or hydroxyl plus methoxyl groups) into this moiety to give the *p*-coumaroyl (or feruloyl) part of the avenanthramides. The percentage of labeled compound in the individual avenanthramides was estimated from the ratio of the ion intensity of $M^+ + 5$ (or $M^+ + 4$) to that of $M^+ + 1$ (Table 2). Labeled compounds accounted for ca. 60–75% of individual avenanthramides. These values were identical to those calculated from the relative intensity of fragment ion peaks de-

Table 2
Incorporation of deuterium into avenanthramides from [2,3,4,5,6-$^{2}$H]L-phenylalanine

| Avenanthramide | Amount (nmol) | Percentage of labelled compound | Incorporation (%) |
|---|---|---|---|
| A | 1503 | 74 (72[a]) | 11.1 |
| B | 298 | 67 (64[a]) | 2.00 |
| D | 40 | 73 (74[a]) | 0.29 |
| G | 104 | 75 (76[a]) | 0.78 |
| L | 237 | 63 (64[a]) | 1.49 |

[a]Percentage of labeled compound determined from the region of the fragment ion produced by the loss of anthranilic moiety.

Table 3
Ion intensity of the molecular ion region of he mass spectra of avenanthramides produced by oat leaf segments treated with [2,6-$^2$H]L-tyrosine. The values in parentheses are theoretical abundance of $[M + H]^+$ ion species calculated from natural abundance of $^{13}C$, $^{15}N$ and $^{18}O$

| | Avenanthramide | | | | |
|---|---|---|---|---|---|
| | A | B | D | G | L |
| $M^+ + 1$[a] | 100 (100) | 100 (100) | 100 (100) | 100 (100) | 100 (100) |
| $M^+ + 2$ | 15.8 (18.2) | 22.0 (19.3) | 17.1 (18.1) | 17.2 (18.2) | 20.7 (20.4) |
| $M^+ + 3$ | 3.1 (2.6) | 3.5 (3.0) | 2.1 (2.3) | 2.6 (2.6) | 3.2 (3.0) |

[a]Intensity of $[M + H]^+$ ion was arbitrarily set to 100%.

rived by the loss of the anthranilic moiety. The incorporation yields were determined individually from the amount of avenanthramides produced and the percentage of labeled compound. In compound **1**, about 11% of administered [2,3,4,5,6-$^2$H]L-phenylalanine was incorporated.

In compound **5**, which has avenalumic acid instead of an hydroxycinnamic acid as in other avenanthramides, incorporation of four deuterium atoms was also observed. This result indicated that the avenalumic acid in **5** was derived from phenylalanine. The percentage of labeled compound in **5** (63%) was slightly lower than that of other avenanthramides, probably due to the longer biosynthetic pathway of **5**.

By the same method used for phenylalanine, administration of [2,6-$^2$H]L-tyrosine was investigated. However, in all avenanthramides produced by oat leaf segments in the presence of [2,6-$^2$H]L-tyrosine, only a very little increase in the $M^+ + 3$ ion peaks was observed by LC-MS analyses (Table 3), suggesting that tyrosine was poorly incorporated into avenanthramides.

### 2.3. Incorporation of [1,2-$^{13}$C]p-coumaric acid

Incorporation of phenylalanine into avenanthramides suggests that biosynthesis of avenanthramides is closely related to phenylpropanoid metabolism. To confirm this, [1,2-$^{13}$C]*p*-coumaric acid was administered to oat leaf segments treated with penta-*N*-acetylchitopentaose. After 24 h incubation, avenanthramides secreted into elicitor soln were analyzed by LC-MS. In all avenanthramides observed, the intensity of the $M^+ + 3$ ion species was larger than the theoretical abundance of $M^+ + 3$ ion species calculated from the natural presence of $^{13}C$, $^{15}N$ and $^{18}O$, indicating that two $^{13}C$ atoms were incorporated into avenanthramides from labeled *p*-coumaric acid. As summarized in Table 4, the percentages of labeled compounds in individual avenanthramides were in the range of 20–30%. These results indicated that the hydroxycinnmamoyl part of all avenanthramides was probably derived from phenylalanine via *p*-coumaric acid. The finding that two $^{13}C$ atoms were incorporated from [1,2-$^{13}$C]*p*-coumaric acid into compound **5**, as they are into other avenanthramides **1**–**4**, also suggested that avenalumic acid is biosynthesized by the elongation of the side chain of *p*-coumaric acid. This would predict that the carbons at 3- and 4-position of avenalumic acid are derived from those at the 1- and 2-positions of *p*-coumaric acid.

### 2.4. Feeding of sodium [$^{13}C_2$]acetate

To examine whether the side chain of *p*-coumaric acid is elongated by an acetate unit to give avenalumic acid, we investigated incorporation of sodium [$^{13}C_2$]acetate into **5**. After a 24 h incubation of oat leaf segments with the elicitor in the presence of sodium [$^{13}C_2$]acetate, avenanthramides liberated into elicitor soln were analyzed by LC-MS. As can be seen in Table 5, compound **5** containing two $^{13}C$ atoms from sodium [$^{13}C_2$]acetate represented 33.8% of the total amount of **5**, whereas in other avenanthramides, less than 2% of compounds contained two $^{13}C$ atoms. The fragment ion peak at $m/z$ 173 derived from the mol-

Table 4
Incorporation of $^{13}C$ into avenanthramides from [1,2-$^{13}$C]*p*-coumaric acid

| Avenanthramide | Amount (nmol) | Percentages of labelled compound | Incorporation (%) |
|---|---|---|---|
| A | 1224 | 24 | 2.9 |
| B | 106 | 20 | 0.21 |
| D | 138 | 3 | 0.46 |
| G | 113 | 31 | 0.36 |
| L | 31 | 20 | 0.06 |

Table 5
Incorporation of $^{13}C$ into avenanthramides from sodium [$^{13}C_2$]acetate

| No. of $^{13}C$ atoms incorporated from sodium [$^{13}C_2$]acetate | Avenanthramide (%) | | | | |
|---|---|---|---|---|---|
| | A | B | D | G | L |
| 0 | 95.8 | 93.2 | 92.2 | 91.9 | 59.1 |
| 1 | 3.7 | 6.6 | 6.0 | 8.0 | 4.9 |
| 2 | 0.5 | 0.2 | 1.8 | 0.1 | 33.8 |
| 3 | n.d.[a] | n.d. | n.d. | n.d. | 2.2 |

[a]n.d., not detectable.

ecular ion by the loss of the anthranilic moiety was also accompanied by a peak at *m/z* 175. Intensities of these fragment ion peaks showed that 33.9% of the **5**-derived fragment originated from [$^{13}C_2$]acetate, which was compatible with the value estimated from the isotope ratio of the molecular ion.

## 3. Discussion

In most plants, induction of phenylpropanoid phytoalexins is the result of the activation of biosynthetic enzymes such as phenylalanine ammonia-lyase and chalcone synthase (Kuc, 1995). However, in several leguminous plants, release of phytoalexins or their precursors from glycosides has been reported (Graham, Kim, & Graham, 1990; Barz & Welle, 1992). To establish that oat phytoalexins, avenanthramides, are de novo synthesized from primary metabolites, labeled putative precursors were administered such as [ring-UL-$^{14}C$]anthranilic acid, [2,3,4,5,6-$^{2}H$]L-phenylalanine and [2,6-$^{2}H$]L-tyrosine, into elicitor-treated oat leaf segments. Among them, anthranilic acid and phenylalanine were incorporated into avenanthramides with low dilution of isotopes. The percentages of labeled compounds in avenanthramides administered labeled phenylalanine were in the range of 60–70%, but there was no indication of incorporation of tyrosine into avenanthramides. These findings strongly suggest that the induction of avenanthramide accumulation in oats by penta-*N*-acetylchitopentaose is the result of de novo synthesis from anthranilic acid and phenylalanine. In addition, although the incorporation yield was lower than that of phenylalanine, *p*-coumaric acid was also incorporated, which indicated that the hydroxycinnamoyl moieties of avenanthramides were biosynthesized via the general phenylpropanoid pathway.

Together with retention of four deuterium atoms of [2,3,4,5,6-$^{2}H$]L-phenylalanine in **5**, incorporation of two $^{13}C$ atoms was observed from both [$^{13}C_2$]sodium acetate and [1,2-$^{13}C$]*p*-coumaric acid, indicating that the avenalumoyl moiety of **5** is biosynthesized from *p*-coumaric acid and one acetate unit. On this basis, the following biosynthetic pathway to avenalumic acid is proposed: condensation of *p*-coumaroyl-CoA and malonyl-CoA to form 5-(4-hydroxyphenyl)-3-oxo-4-pentenoyl-CoA; then this intermediate is reduced and dehydrated to avenalumoyl-CoA (Fig. 1). Based on the

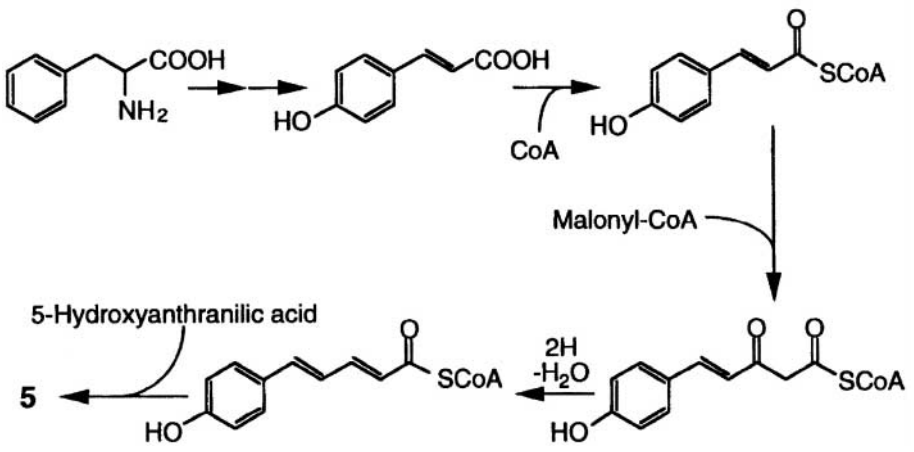


Fig. 1. Proposed biosynthetic pathway to avenanthramide L.

pivotal role of hydroxycinnamoyl-CoA thioesters in phenylpropanoid metabolism in many plant species (Zenk & Gross, 1972; Hahlbrock & Grisebach, 1979), the elongated side chain is thought to be formed by condensation between *p*-coumaroyl-CoA and malonyl-CoA, although no evidence of the involvement of CoA thioesters in this process has been obtained to date. This mechanism is the same as that supposed for biosynthesis of piperic acid (5-(3,4-methylenedioxyphenyl)-2,4-pentadienoic acid), a constituent of the pungent principle of pepper, piperine (Gross, 1981). To our knowledge, incorporation of labeled *p*-coumaric acid and sodium acetate into **5** is the first evidence of this mechanism for biosynthesis of the 5-phenyl-2,4-pentadienoic acid structure in plants.

## 4. Experimental

### 4.1. Plant materials

Oat seeds (*Avena sativa* L., cv. Shokan 1) were soaked in $H_2O$ at room temp. for 12 h. The soaked seeds were then sown in wet vermiculite, and maintained at 20°C for seven days under continuous fluorescent lights (40 W $m^{-2}$) in growth chambers.

### 4.2. Chemicals

Avenanthramide A (*N*-(4-hydroxycinnamoyl)-5-hydroxyanthranilic acid, **1**), avenanthramide B (*N*-(3-methoxy-4-hydroxycinnamoyl)-5-hydroxyanthranilic acid, **2**) and avenanthramide D (*N*-(4-hydroxycinnamoyl)anthranilic acid, **3**) were synthesized according to the method of Collins (1989). The synthesis of avenanthramide G (*N*-(4-hydroxycinnamoyl)-4-hydroxyanthranilic acid, **4**) and L (*N*-[5-(4-hydroxyphenyl)-(2*E*,4*E*)-2,4-pentadienoyl]-5-hydroxyanthranilic acid, **5**) have been reported previously (Miyagawa et al., 1995, 1996a). [ring-UL-$^{14}$C]Anthranilic acid (462.5 MBq/mmol) was purchased from Sigma. [2,3,4,5,6-$^{2}$H]L-Phenylalanine (98 at.% of $^{2}H_5$), [2,6-$^{2}$H]L-tyrosine (98 at.% of $^{2}H_2$), sodium [$^{13}C_2$]acetate (99 at.% of $^{13}C_2$) and [$^{13}C_3$]malonic acid (99 at.% of $^{13}C_3$) were purchased from Isotec, USA. [1,2-$^{13}$C]*p*-Coumaric acid was synthesized according to Freudenberg and Fuchs (1954) with slight modification. [$^{13}C_3$]Malonic acid (0.1 g, 0.93 mmol) was dissolved in dry pyridine with 4-hydroxybenzaldehyde (0.12 g, 0.98 mmol). After adding piperidine (0.1 ml, 1.0 mmol) and aniline (0.1 ml, 1.2 mmol), the reaction mixture was incubated in boiling water for 2 h. After evaporation of pyridine, the residue was dissolved in water followed by acidification with 1 N HCl. The soln was fractionated on an ODS column (YMC ODS-AM 120-S50, $10 \times 250$ mm) that was equilibrated with $H_2O$ containing 2% HOAc. The column was eluted stepwise with 100 ml each of 20%, 50% and 80% MeOH in $H_2O$ containing 2% HOAc. *p*-Coumaric acid was detected in the 50% MeOH fraction. This fraction was concentrated in vacuo and subjected to purification by HPLC (column, Wakosil II 5C18-HG $25 \times 250$ mm; solvent, 30% MeOH in $H_2O$ containing 0.1% HOAc; flow rate, 0.8 ml $min^{-1}$; detection, UV 280 nm) to give powdery crystals (0.105 g, 64%). $^1$H NMR (300 MHz, $CD_3OD$) $\delta$: 6.27 (1H, ddd, $^1J_{HC} = 160.5$ Hz, $^3J_{HH} = 15.9$ Hz, $^2J_{HC} = 2.8$ Hz), 6.80 (2H, d, $J = 8.6$ Hz), 7.44 (2H, d, $J = 8.6$ Hz), 7.60 (1H, ddd, $^3J_{HH} = 15.9$ Hz, $^2J_{HC} = 6.71$ Hz, $^2J_{HC} = 2.8$ Hz). $^{13}$C NMR (75 MHz, $CD_3OD$) $\delta$: 115.6 ($^1J_{2-1} = 73.2$ Hz, $^1J_{2-3} = 70.1$ Hz), 116.7, 127.2 ($^3J_{1'-1} = 6.9$ Hz), 131.0 ($^3J_{2'-2} = 4.7$ Hz), 146.6 ($^1J_{3-2} = 70.1$ Hz), 161.0, 170.9 ($^1J_{1-2} = 73.2$). Ion-spray MS *m*/*z* (rel. int.): 189 (12.3), 167 (30.8), 149 (100), 120 (54.9). The at.% of $^{13}C_2$ of [1,2-$^{13}$C]*p*-coumaric acid was 98.1%, which was determined by ion intensities in the $[M + H]^+$ region of the ion-spray MS spectrum.

### 4.3. Administration of [ring-UL-$^{14}$C]anthranilic acid

The lower epidermis of 7 day old primary oat leaves was peeled off and 5 mm segments taken 1–6 cm from the leaf tip. Three segments (50 mg fr.wt.) were floated on 300 μl of 1 mM penta-*N*-acetylchitopentaose (Seikagaku Kogyo, Japan) aq. soln containing 0.57 mM [ring-UL-$^{14}$C]-anthranilic acid. After a 24 h incubation at 20°C, 20 μl of elicitor soln was subjected to HPLC equipped with an ODS column (Wakosil II 5C18 HG $4.6 \times 150$ mm). For purification of avenanthramides, two different solvent systems were used at a flow rate of 0.8 ml/min; system 1: 48% MeOH in $H_2O$ containing 0.5% TFA for **1** and **2**, and system 2: 55% MeOH in $H_2O$ containing 0.5% TFA for **3**, **4** and **5**. Avenanthramides were detected at 340 nm. Radioactivity in individual avenanthramides was determined by liquid scintillation counting after collection of the peaks.

### 4.4. Administration of precursors labeled with stable isotopes

The lower epidermis of 7 day old primary oat leaves was peeled off and 5 cm segments taken 1–6 cm from the leaf tip. Twenty leaf segments (650 mg fr.wt.) were floated on 10 ml of 1 mM penta-*N*-acetylchitopentaose aq. soln containing 1 mM labeled compounds. After incubation for 24 h at 20°C, the elicitor soln was loaded on a Sep-pak plus C18 cartridge (Waters) equilibrated with 20% MeOH in $H_2O$ containing 2% HOAc. After washing with 5 ml of 20% MeOH in $H_2O$ containing 2% HOAc, the cartridge was eluted with 5 ml of 80% MeOH in $H_2O$ containing 2%

HOAc. The 20–80% MeOH fraction containing avenanthramides was concentrated to dryness in vacuo, and the residue was dissolved in 0.5 ml of MeOH. This soln was subjected to ion-spray LC-MS analyses. The positive ion-spray ionization mass spectra measurements were carried out using a Parkinermer-Sciex API-165 instrument (ion-spray voltage: 5 kV, orifice voltage: 30 V, nebulizer gas: air, curtain gas: nitrogen) combined with a Shimadzu 10A HPLC system equipped with an ODS column (Wakosil II 5C18 HG, $4.6 \times 150$ mm). The following HPLC conditions were used: solvent: 55% MeOH in $H_2O$ containing 0.1% TFA, flow rate: 0.8 ml/min. After passing through the column, the eluent was split, and 5% of it was introduced to the MS instrument. For measurement of $[M + H]^+$ ion abundance, the scanning mass range was set to $M-1$–$M+9$. Percentages of compounds containing stable isotopes in individual avenanthramides were calculated from the ion intensity in the $[M + H]^+$ peak region taking into consideration the natural abundance of $^{13}C$, $^{15}N$ and $^{18}O$.

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