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INDUCTION OF HYDROXYANTHRANILATE HYDROXYCINNAMOYL TRANSFERASE ACTIVITY BY OLIGO-*N*-ACETYLCHITOOLIGOSACCHARIDES IN OATS

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Key Word Index—*Avena sativa*; Gramineae; avenanthramide; oats; anthranilic acid; hydroxycinnamoyl-CoA; biosynthesis.

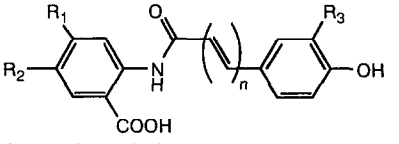
Abstract—An assay method for hydroxycinnamoyl-CoA: hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT) in oat leaves (*Avena sativa* L.), which is thought to be one of the key enzymes for the biosynthesis of avenanthramides, phytoalexins in this plant, was established. HHT activity was induced by treating the leaves with oligo-*N*-acetylchitooligosaccharides. Among the chitooligosaccharides tested, penta-*N*-acetylchitopentaose ((GlcNAc)$_5$) was the most effective in inducing activity. The induction by (GlcNAc)$_5$ was dose-dependent, in which case HHT activity was initially detected after 6 hr and reached a maximum by 12 hr. All of the putative precursors of avenanthramides acted as substrates for HHT, with 5-hydroxyanthranilic acid and feruloyl-CoA being the best substrates for the anthranilic moiety and the cinnamoyl moiety of avenanthramides, respectively.

INTRODUCTION

Higher plants express a variety of resistance responses to parasites, including fungal pathogens. Among these, the production of antifungal substances, such as phytoalexins is thought to be important in preventing the growth of microorganisms at the site of the attempted infection.

In oats (*Avena sativa* L.), the production and accumulation of phytoalexins was demonstrated, following the inoculation of a pathogen, crown rust fungus (*Puccinia coronata* f. sp. *avenae*) in incompatible combinations [1]. These phytoalexins have been shown to be a series of substituted *N*-cinnamoylanthranilates, which are referred to as avenanthramides (**1**–**5**) [2]. Structurally-analogous phenolic acid amides have also been found in other higher plants, some of which have been demonstrated to be related to stress responses [3–6], and are synthesized from phenolic acid-CoA thioesters and the corresponding amines in plant tissues.

Phytoalexins in oats can also be produced by treatment of leaves with various elicitors, such as chitin and chitosan oligomers [7], victorin C [8], heavy metals [9] and calcium ionophore A23187 [10]. Among these elicitors, the mode of action of chitin oligomers is of particular interest, since the cell walls of the crown rust fungus are composed largely of chitin and glucan [11, 12]. The present study shows that the treatment of oat leaves with oligo-*N*-acetylchitooligosaccharides induces hydroxycinnamoyl-CoA: hydroxyanthranilate *N*-hydroxycinnamoyl transferase (HHT) activity which catalyses the final reaction in the putative route for phytoalexin biosynthesis in oats.



1: Avenanthramide A, R_1=H, R_2=OH, R_3=H, n=1
2: B, R_1=H, R_2=OH, R_3=MeO, n=1
3: D, R_1=H, R_2=H, R_3=H, n=1
4: G, R_1=OH, R_2=H, R_3=H, n=1
5: L, R_1=OH, R_2=H, R_3=H, n=2

RESULTS

Detection of HHT activity

Oat leaf segments were treated with (GlcNAc)$_5$ at 1 mM for 12 hr. On incubation of the crude extract from the treated leaf segments with *p*-coumaroyl-CoA and 5-hydroxyanthranilic acid for 60 min at 30°, the

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formation of **1** was detected (24.5 nmol reaction mixture^{-1}). When the leaves were treated with H_2O for 12 hr, the extract exhibited only a slight activity for the formation of **1** (2.35 nmol reaction mixture^{-1}). The protein contents in the crude extracts from elicitor- and H_2O-treated leaves were 8.17 and 8.41 mg ml^{-1}, respectively. No amide was formed in the absence of the leaf extract, nor in the presence of boiled extract.

Induction of HHT by oligo-N-acetylchitooligosaccharides

In order to establish analytical conditions for the assay of HHT activity, the formation of **1** in the reaction mixture was examined. The enzyme solution was extracted with 10 volumes of 0.1 M Na-Pi buffer, 12 hr after treatment of the oat leaves with elicitor. The amount of **1** increased linearly up to 30 min, and thereafter the rate of formation gradually decreased. The initial rate of formation was in proportion to the amount of proteins added in the reaction mixture, as the diluted extracts (3- and 9-fold) expressed proportionally low activities. Based on these findings, the enzyme activity was determined by quantifying the product after a 20 min incubation period in the experiments described below.

Time courses for the induction of HHT activity. Figure 1 shows the time courses for changes in HHT activity in oat leaf segments treated with $(GlcNAc)_5$. 5-Hydroxyanthranilic acid and *p*-coumaroyl-CoA were used as substrates. Little HHT activity was detectable at 0 hr, but the activity increased dramatically as the result of elicitor treatment. HHT activity was significantly increased after 6 hr and reached a maximum at 12 hr, and then declined to 50% of the maximal value after 24 hr of treatment. Thereafter, the activity remained constant up to 60 hr. An increase in HHT activity was also observed in the control leaf segments after 60 hr, probably due to damage caused by peeling off the lower epidermis.

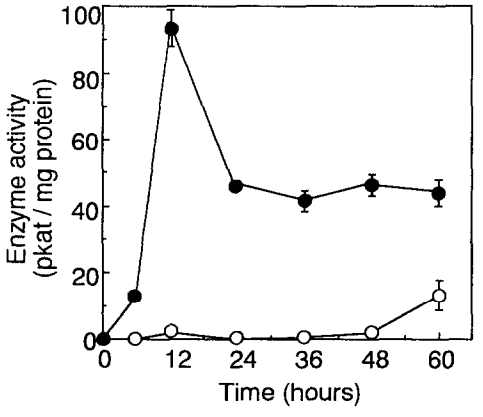

Fig. 1. Changes in HHT activity. 5-Hydroxyanthranilic acid and *p*-coumaroyl-CoA were used as substrates. At time 0, oat leaf segments were floated in 1 mM $(GlcNAc)_5$ solution (●) or distilled H_2O (○) and incubated at 20° under artificial light. HHT activity is plotted as a function of time after the initiation of elicitor treatment. The results are expressed as the means of triplicate experiments with ±SD.

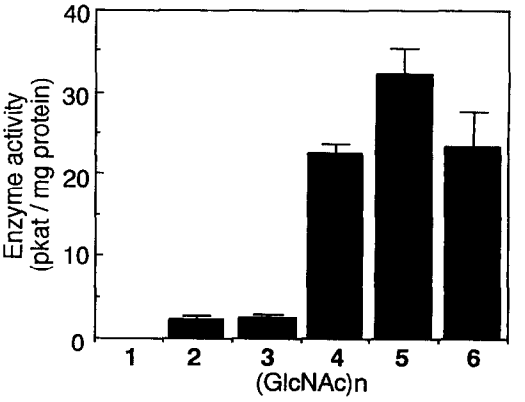

Fig. 2. Effects of the degree of polymerization of oligo-*N*-acetylchitooligosaccharides on the induction of HHT activity. Oat leaf segments were floated in various oligo-*N*-acetylchitooligosaccharide solutions at a concentration 1 mM. After 12 hr incubation at 20° in the presence of light, the segments were extracted and HHT activity determined. HHT activity was measured using 5-hydroxyanthranilic acid and *p*-coumaroyl-CoA as substrates. The results are expressed as the means of triplicate experiments with ±SD.

Effect of the degree of polymerization of oligo-N-acetylchitooligosaccharides on the induction of HHT activity. A series of oligo-*N*-acetylchitooligosaccharides, having degrees of polymerization of 1 to 6 was examined, in terms of their ability to induce HHT activity (Fig. 2). The activity was clearly dependent on the degree of polymerization. Saccharides smaller than the triose showed little activity at 1 mM concentrations, while the pentasaccharide was most effective in inducing HHT activity. The tetra- and hexasaccharides also showed potent elicitor activities, although they were somewhat less active than the pentasaccharide.

Effect of elicitor concentration. Figure 3 shows the induction of HHT activity in oat leaf segments treated with $(GlcNAc)_5$ at different concentrations, using *p*-coumaroyl-CoA and 5-hydroxyanthranilic acid as substrates. The induced HHT activity increased sharply with an increase in the added elicitor, up to 0.25 mM. At 4 mM, the activity was reduced to 70% of the maximum value.

Properties of the HHT activity induced by $(GlcNAc)_5$

In order to study the dependence on pH and the substrate specificity of the induced HHT activity, a crude extract of the leaf segments, prepared after 12 hr of elicitor treatment, was partially purified by salting-out with $(NH_4)_2SO_4$. Sixty percent of the initial activity was recovered in the fraction which precipitated between 30 and 45% saturation. By storing at −30° after addition of glycerol, the enzyme solution could be preserved for 1 month with a 10% activity loss.

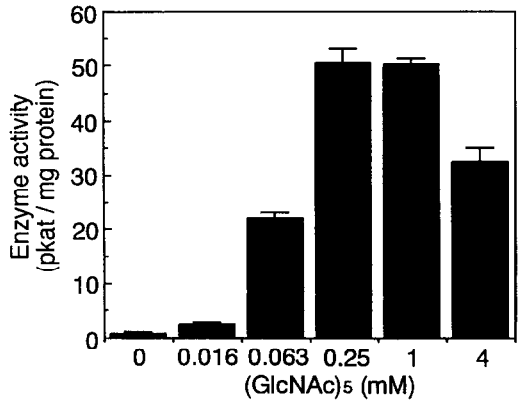

Fig. 3. Induction of HHT activity by various concentrations of $(GlcNAc)_5$. Oat leaf segments were floated in solutions containing various concentrations of $(GlcNAc)_5$. HHT activity in oat leaf tissue was measured 12 hr after the initiation of elicitor treatment using 5-hydroxyanthranilic acid and *p*-coumaroyl-CoA as substrates. The results are expressed as the means of triplicate experiments with ±SD.

Dependence of HHT activity on pH. HHT activity, with respect to the formation of compound **1**, was detected between pH 5.5 and 10. The maximum activity was observed at pH 7 using 0.1 M Na-Pi buffer, whereas pH 7.5 was the optimum in GTA (0.05 M 3,3-dimethylglutaric acid, 0.05 M Tris, 0.05 M 2-amino-2-methyl-1,3-propanediol) buffer solution.

Substrate specificity of elicitor-induced HHT activity. In view of the fact that five types of avenanthramides (**1**–**5**) are found in the elicitor-treated oat leaves (Table 1), the substrate specificity of HHT activity was investigated. Table 2 shows the elicitor-induced HHT activity in oat leaf segments with various substituted anthranilic acids, with *p*-coumaroyl-CoA being used as the acyl donor. All of the substituted-anthranilic acids, which consist of naturally occurring avenanthramides, acted as substrates, while no conversion was detected for the case of 3-hydroxyanthranilic acid and tyramine. The lowest apparent K_m and the largest V_{max} value was observed for 5-hydroxyanthranilic acid. These results suggest that HHT has a high specificity for the anthranilic moiety.

Table 3 shows the HHT activity in the presence of various cinnamoyl-CoAs. 5-Hydroxyanthranilic acid was used as the acyl acceptor. All of the tested CoA derivatives acted as substrates. Based on V_{max}/K_m values, the best substrate was feruloyl-CoA, followed by avenalumoyl-CoA and cinnamoyl-CoA. Compared with these compounds, the acceptance of *p*-coumaroyl-CoA and caffeoyl-CoA was somewhat lower.

Table 1. Induction of avenanthramides by $(GlcNAc)_5$. Oat leaf segments were floated in 1 mM $(GlcNAc)_5$ solution. Each value represents the mean ± SD from three experiments

Avenanthramides	nmol g fr. wt.$^{-1}$	%
A (**1**)	1323.7 ± 268.7	100
B (**2**)	184.8 ± 13.4	14
D (**3**)	21.6 ± 5.4	1.6
G (**4**)	73.8 ± 16.4	5.6
L (**5**)	88.2 ± 7.0	6.7

The highest reactivity of 5-hydroxyanthranilic acid as an anthranilic substrate corresponded to the largest production of **1**, among the induced avenanthramides in the elicited oat leaves, while the substrate specificity with respect to the acyl moiety did not correlate to the amounts of the products in the leaves (Table 1). In particular, no conjugate with cinnamic acid nor caffeic acid was detected in the elicitor-treated oat leaves under the experimental conditions employed in this study, although their corresponding CoA derivatives were active as HHT substrates.

DISCUSSION

The treatment of oat leaf segments with $(GlcNAc)_5$ markedly induced HHT activity, suggesting that the activity of this enzyme is responsible for phytoalexin production in this plant. All the putative precursors of a series of avenanthramides found in the elicited leaves, acted to a greater or lesser extent, as substrates, suggesting that each of the avenanthramides was individually synthesized by the condensation of the corresponding substituted anthranilic acids and the substituted cinnamoyl-CoA thioesters. This is in contrast with the production of a series of substituted *N*-benzoylanthranilates, referred to as dianthramides, phytoalexins of carnation. Biosynthetic studies of dianthramides have shown that the substrate specificity of amide-forming enzyme is not broad, and that substituents on the aromatic rings, such as hydroxyl and methoxy groups are introduced after amide formation [13]. Similar biosynthetic pathways could also occur in oats for the case of avenanthramide production, since **1** could be formed from **3** by oxidative hydroxylation. Two possible routes for the biosynthesis of **1** are schematically shown in Fig. 4.

The relative substrate specificity of HHT does not necessarily correlate with the amount of the individual avenanthramides induced by $(GlcNAc)_5$ in oat leaves. For example, although HHT had the highest V_{max}/K_m value for feruloyl-CoA, product **2** accounted for only a minor portion of the induced metabolites. This suggests that the amount of accumulated avenanthramides depends largely on the amount of available substrate for HHT in the oat tissue. Alternatively, the discrepancy can be explained by differences in the metabolic fate of each individual avenanthramide. It has been suggested that amine-conjugated phenolic acids, such as dianthramide in carnation [14], hydroxycinnamoyltyramine in potato [4] and feruloyltyramine in tobacco [15], are integrated into the cell walls in the form of insoluble polymeric material. Avenanthramides might be analogously metabolized further in oat tissue.

In addition to chitin oligomers, a variety of elicitors,

Table 2. Substrate specificity of HHT for the anthranilic moiety. *p*-Coumaroyl-CoA (0.5 mM) was used as the common acyl donner

Substrate	$10^5 \times K_m$ (M)	Relative V_{max} (%)	$10^{-5} \times V_{max}/K_m$
5-Hydroxyanthranilate	26	100	3.80
Anthranilate	63	79	1.26
4-Hydroxyanthranilate	100	76	0.76
3-Hydroxyanthranilate	n.d.	n.d.	—
Tyramine	n.d.	n.d.	—

n.d. = not detectable.

Table 3. Substrate specificity of HHT for the cinnamoyl moiety. 5-Hydroxyanthranilic acid (1 mM) was used as the common acyl accepter

Substrate	$10^5 \times K_m$ (M)	Relative V_{max} (%)	$10^{-5} \times V_{max}/K_m$
Feruloyl-CoA	36	100	2.8
Cinnamoyl-CoA	23	30	1.3
p-Coumaroyl-CoA	9.6	7.8	0.81
Caffeoyl-CoA	14	6.5	0.46
Avenalumoyl-CoA	2.1	5.1	2.4
4-Hydroxybenzoyl-CoA	15	1.4	0.093

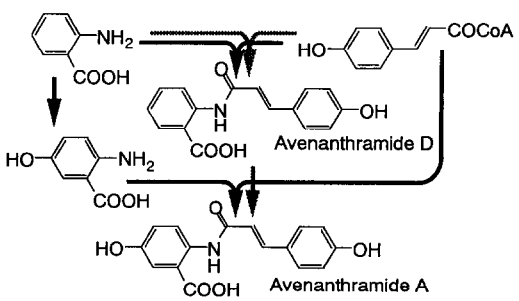

Fig. 4. Proposed biosynthetic pathways of avenanthramides.

such as chitosan oligomers, victorin C, heavy metal ions and calcium ionophore A23187, induce avenanthramides. Recently, it was reported that an elicitor-dependent difference was observed in the composition profile of avenanthramides [16]. The possibility that induction of HHT activity is dependent on elicitors, merits further study.

EXPERIMENTAL

Plant material. Oat seeds (*Avena sativa* L., cv Shokan 1) were soaked in H_2O at room temp. for 12 hr in darkness. The soaked seeds were then sown in wet vermiculite, and maintained at 20° for 7 days under continuous artificial light.

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 ref. [17]. 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 [2, 18].

The method used for the synthesis of avenanthramide C (*N*-(3′,4′-dihydroxycinnamoyl)-5-hydroxyanthranilic acid) was similar to that described in ref. [19] for the prepn of feruloyltyramine. Caffeic acid (2 mmol) was mixed with 5-hydroxyanthranilic acid (1 mmol), and dicyclohexylcarbodiimide (2.2 mmol) in anhydrous pyridine (10 ml). After 24 hr, the pyridine was evapd *in vacuo*. The residue was then dissolved in 25 ml MeOH and cooled on an ice bath. After filtration, 25 ml 1 M KOH was added to the filtrate and the mixt. stirred for 24 hr at room temp. The MeOH was then evapd *in vacuo* and avenanthramide C was extracted in EtOAc, after neutralization of the KOH with HOAc. The ODS HPLC system (Wakosil II 5C18HG column; mobile phase of 45% MeOH in H_2O containing 0.5% TFA) was used to purify avenanthramide C. The other reference amide compounds, *N*-(4′-hydroxycinnamoyl) tyramine, *N*-(4′-hydroxybenzoyl)-5-hydroxyanthranilic acid, *N*-cinnamoyl-5-hydroxyanthranilic acid and *N*-(4′-hydroxycinnamoyl)-3-hydroxyanthranilic acid were similarly synthesized. The spectroscopic data of the prepared compounds are as follows:

N-(4′-Hydroxybenzoyl)-5-hydroxyanthranilic acid. ^{1}H NMR δ (MeOH-d_4): 6.91 (2H, *d*, 8.7), 7.06 (1H, *dd*, 9.0, 3.0), 7.44 (1H, *d*, 3.0), 7.79 (2H, *d*, 8.7), 8.52 (1H, *d*, 9.0). EI-MS *m/z*: 273 $[M]^+$, 255, 153, 121, 93, 65.

N-Cinnamoyl-5-hydroxyanthranilic acid. ^{1}H NMR δ (MeOH-d_4): 6.72 (1H, *d*, 15.7), 7.03 (1H, *dd*, 9.0, 3.0), 7.38–7.43 (3H, *m*), 7.52 (1H, *d*, 3.0), 7.62 (2H, *dd*, 7.7, 2.3), 7.65 (1H, *d*, 15.7), 8.46 (1H, *d*, 9.0). EI-MS *m/z*: 283 $[M]^+$, 265, 264, 153, 131, 103, 77.

N-(4′-Hydroxycinnamoyl)-3-hydroxyanthranilic acid. ^{1}H NMR δ (MeOH-d_4): 6.70 (1H, *d*, 15.5), 6.84 (2H, *d*, 8.6), 7.16–7.21 (2H, *m*), 7.52 (2H, *d*, 8.6), 7.61 (1H, *dd*, 7.1, 2.2), 7.67 (1H, *d*, 15.5). EI-MS *m/z*: 299 $[M]^+$, 281, 254, 147, 131.

The spectroscopic data of other standard samples have been previously described [2, 4, 17, 18].

Hydroxycinnamoyl- and hydroxybenzoyl-CoA thioesters were prepd by transesterification of hydroxycinnamoyl- and hydroxybenzoyl-*N*-hydroxysuccinimide esters, as described in ref. [20]. Concns of the obtained CoA derivatives were calcd and adjusted on the basis of known molar extinction coefficients [20, 21] except for avenalumoyl-CoA. Avenalumoyl-CoA was quantified by HPLC analysis of avenalumic acid liberated from CoA thioester by alkaline treatment (30 min, 0.1 M NaOH at room temp.). 4-Hydroxyanthranilic acid was synthesized from 2,4-dinitrobenzoic acid according to the method of ref. [13].

Elicitor treatment and induction of avenanthramides. 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. The segments were floated on 10 ml of elicitor soln in a Petri dish with the peeled surface in contact with the soln. Concns of oligo-*N*-acetylchitooligosaccharides at 1 mM were used as elicitors. The amounts of induced avenanthramides were determined by HPLC analysis of the elicitor soln after 24 hr elicitation. Most of the avenanthramides produced by oat leaf segments are secreted in the soln [16].

Enzyme extraction and assay. Enzyme extraction was carried out at 0–4°. Frozen oat leaf segments (*ca* 350 mg) were ground in 2 vol. 0.1 M Na-Pi buffer (pH 7.5) for detection of HHT activity. After centrifugation (12 000 *g*, 10 min, 4°), the supernatant was used as the crude enzyme soln. The reaction mixt. consisted of 25 μl of crude enzyme, 20 μl of 10 mM 5-hydroxyanthranilic acid, 15 μl of 0.5 mM *p*-coumaroyl-CoA and 40 μl of 0.1 M Na-Pi buffer at pH 7.5. After 60 min incubation at 30°, the reaction was stopped by adding 20 μl of HOAc. The mixt. was then filtered, followed by the addition of 380 μl of MeOH to remove the ppt., and a 10 μl aliquot was analysed by HPLC. Protein content was determined according to the method of ref. [22].

For examining the elicitor activity of oligo-*N*-acetylchitooligosaccharides, the enzyme was extracted by grinding the frozen oat leaf segments in 10 vol. 0.1 M Na-Pi buffer at pH 7.5 containing 14.4 mM 2-mercaptoethnol (ME). After centrifugation (12 000 *g*, 10 min, 4°), the supernatant was used as the enzyme soln. The reaction mixt. consisted of 10 μl of the enzyme, 10 μl of 10 mM 5-hydroxyanthranilic acid, 10 μl of 0.5 mM *p*-coumaroyl-CoA and 70 μl of 0.1 M Na-Pi buffer at pH 7. After 20 min incubation at 30°, the reaction was stopped by adding 20 μl of HOAc, and the products analysed by HPLC as described above.

For determination of the pH optimum and substrate specificity, the enzyme soln was further fractionated by adding $(NH_4)_2SO_4$ (30–45%). The ppt. was desalted on a Sephadex G-25, equilibrated with 0.02 M Na-Pi buffer (pH 7, 14.4 mM ME). After adding 1 vol. glycerol, the enzyme soln was stored at −30° prior to use. The stored enzyme was diluted with 0.02 M Na-Pi buffer (pH 7) and glycerol (1:1) for reactions. The reaction period was set at 10 min. In experiments relative to the pH optimum, the following buffers were used: 0.1 M Na-Pi (pH 6–8); GTA (pH 5–10).

HPLC conditions. The quantity of avenanthramides and other phenolic acids conjugated with amines was determined by HPLC, using an ODS column (Wakosil II 5C18HG 4.6 × 150 mm). Four different mobile phases were used; (1) 48% MeOH in H_2O containing 0.5% TFA for the analysis of **1**, **2**, **4**, and *N*-(4′-hydroxycinnamoyl)-3-hydroxyanthranilic acid, (2) 53% MeOH in H_2O containing 0.5% TFA for **3**, **5** and *N*-cinnamoyl-5-hydroxyanthranilic acid, (3) 43% MeOH in H_2O containing 0.5% TFA for avenanthramide C and *N*-(4′-hydroxybenzoyl)-5-hydroxyanthranilic acid, (4) 40% MeOH in H_2O containing 0.5% TFA for *N*-(4′-hydroxycinnamoyl)tyramine. All phenolic acids conjugated with amines, except the tyramine, were monitored at 340 nm. *N*-(4′-hydroxycinnamoyl) tyramine was monitored at 300 nm.

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