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Stimulatory effect of cinnamic acid analogues on α_{1A}-adrenoceptors in-vitro

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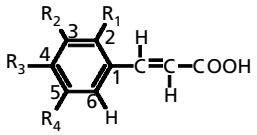
Abstract

We have characterized the effects of cinnamic acid and its derivatives on α_1-adrenoceptor subtypes. The cinnamic acid with a methoxyl group and/or a hydroxyl group showed the ability to stimulate radioactive glucose uptake into C_2C_{12} cells, a cell line that specifically expresses the α_{1A}-adrenoceptor subtype of α_1-adrenoceptors. However, cinnamic acid without chemical modification diminished the glucose uptake into C_2C_{12} cells. It was shown that methoxylation and/or hydroxylation of cinnamic acid had higher affinities for α_{1A}-adrenoceptors investigated using [^{3}H]prazosin binding experiments in C_2C_{12} cells. The effect of these derivatives on α_{1A}-adrenoceptors was further characterized using the displacement of [^{3}H]prazosin binding in rat prostate. We found that 3,5-dimethoxy-4-hydroxycinnamic acid, the cinnamic acid derivative with two methoxyl groups and hydroxylation at the fourth carbon on the benzene ring, had a higher affinity for the α_{1A}-adrenoceptor subtype, showing a smaller IC50 value (the concentration for production of 50% inhibition) to displace [^{3}H]prazosin binding in rat prostate. Affinity of these compounds for α_{1B}-adrenoceptors was identified using [^{3}H]prazosin-binding experiments in rat spleen. However, we found no marked differences in the IC50 values between these cinnamic acid analogues to displace the [^{3}H]prazosin binding in rat spleen. In conclusion, our data indicated that methoxylation and/or hydroxylation of cinnamic acid might raise the affinity for α_{1A}-adrenoceptors.

Introduction

Adrenoceptors, belonging to the G-protein-linked family of receptors, respond to the physiological agonists noradrenaline and adrenaline in noradrenergic neurotransmission. Multiple α_1-adrenoceptor subtypes have been documented (Morrow & Creese 1986; Lomasney et al 1991). Recently, the α_1-adrenoceptor was classified into three main subtypes, termed α_{1A}-, α_{1B}-, and α_{1D}-, and their cloned counterparts designated α_{1a}-, α_{1b}-, and α_{1d}- (Hieble et al 1995; Zhong & Minneman 1999). It has been established that a blocker of α_{1A}-adrenoceptors which has less effect on blood pressure is available in the treatment of prostatic hypertrophy (Beduschi et al 1998). Also, the α-adrenergic mechanism is responsible for the glucose transport stimulated by catecholamines in skeletal muscle, the primary tissue involved in glucose utilization in the postprandial state in-vivo (Saitoh et al 1974). Additionally, α_{1A}-adrenoceptors are involved in the regulation of glucose uptake into the white adipocyte (Cheng et al 2000).

The fruit of *Xanthium strumarium* (Compositae), named as Chang′ErZi or Chang′ErChao in Chinese (Cai 1995), has been used in traditional Chinese medicine to prevent human low-density lipoprotein oxidation (Nardini et al 1995). Previously, we reported that caffeic acid (3,4-dihydroxycinnamic acid), a derivative of cinnamic acid, was an active principle isolated from the fruit of *X. strumarium* found to enhance glucose uptake by activating α_{1A}-adrenoceptors in cultured C_2C_{12} cells derived from mouse fast-twitch skeletal muscle (Cheng & Liu 2000). Also, we found that stimulation of α_{1A}-adrenoceptors by isoferulic acid (3-hydroxy-4-methoxycinnamic acid) enhanced the glucose uptake into C_2C_{12} cells (Liu et al 2001). There might by a relationship between compounds in the basic structure of caffeic acid and the activation of α_{1A}-adrenoceptors.



	R_1	R_2	R_3	R_4
Cinnamic acid	H	H	H	H
2-Hydroxycinnamic acid	OH	H	H	H
3-Hydroxy-4-methoxycinnamic acid	H	OH	OCH_3	H
4-Hydroxy-3-methoxycinnamic acid	H	OCH_3	OH	H
3,5-Dimethoxy-4-hydroxycinnamic acid	H	OCH_3	OH	OCH_3
2,5-Dimethoxycinnamic acid	OCH_3	H	H	OCH_3
3,4-Dimethoxycinnamic acid	H	OCH_3	OCH_3	H

Figure 1 The chemical structures of cinnamic acid derivatives.

It is well known that the α_1-adrenoceptor in rat prostate is almost exclusively of the α_{1A}-adrenoceptor subtype (Hiraoka et al 1999). We have demonstrated that α_{1A}-adrenoceptor was the major subtype of α_1-adrenoceptors in the C_2C_{12} cell line (Liu et al 2000). With regards to α_{1B}-adrenoceptors, another subtype of the α_1-adrenoceptor, rat spleen has been used widely to characterize the pharmacological and signalling properties of the α_{1B}-adrenoceptor (Zhong & Minneman 1999). In an attempt to elucidate the effect of cinnamic acid analogues (Figure 1) on α_1-adrenoceptors, the glucose uptake into C_2C_{12} cells and the displacement of [^{3}H]prazosin binding in C_2C_{12} cells, prostate or spleen were performed.

Materials and Methods

C_2C_{12} cell culture

The C_2C_{12} cells were obtained from the Culture Collection and Research Center (CCRC 60083) of the Food Industry Institute (Hsin-Chiu City, Taiwan). The cells were plated at 5×10^4 cells per dish in 35-mm diameter culture dishes in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% foetal bovine serum (FBS) and 1% antibiotic solution (penicillin G sodium 10 000 U mL^{-1} and streptomycin sulfate 10 μg mL^{-1}), and were grown to 70% confluence at 37 °C in a humidified atmosphere containing 5% CO_2. To induce fusion, confluent cells were exposed to DMEM supplemented with 10% horse serum instead of FBS. Cells fused into multinucleated myotubes after a further 7–10 days in culture (Sheriff et al 1992). Medium was changed 24 h before experimental manipulations.

Glucose uptake

The uptake of 2-[^{14}C]deoxy-D-glucose (2-DG) into C_2C_{12} cells was determined as described by Cheng & Liu (2000). In brief, cells were washed with phosphate-buffered saline (PBS) containing (in mmol L^{-1}): 135 NaCl, 2.7 KCl, 8 Na_2HPO_4, 1.4 KH_2PO_4, 0.5 $MgCl_2$, 0.7 $CaCl_2$ and 22 glucose. After 5-h incubation in the serum-free and high glucose (25 mmol L^{-1}) DMEM medium, the cells were transferred to fresh incubation flasks with or without analogues of cinnamic acid or porcine insulin monocomponent at indicated concentrations at 37 °C for 30 min under continuous shaking at 40 cycles min^{-1}. The cells were then further incubated with 2-DG (1 μCi mL^{-1}) for 5 min at 37 °C. Uptake was terminated by addition of ice-cold PBS. After centrifugation, cells were washed twice with ice-cold PBS. Cell-associated radioactivity was determined by lysing the cells in 1 mol L^{-1} NaOH and the samples were neutralized for estimation in a scintillation counter. Nonspecific uptake of 2-DG, assessed after an incubation with 20 μmol L^{-1} cytochalasin B to block transportation, was subtracted from the total cell-associated radioactivity. Specific 2-DG uptake was expressed as the percentage of basal uptake that was obtained from cells incubated with DMEM only.

Radioligand binding

Preparation of the prostate or spleen membrane of rat was performed as described previously (Sladeczek et al 1989; Yazawa et al 1992). Male Wistar rats (220–250 g) were obtained from the animal center of the National Cheng Kung University Medical College. In brief, rats were killed, and the prostate and spleen were immediately removed. Tissues were finely minced with scissors and homogenized in 10 ice-cold volumes homogenization buffer (0.25 mol L^{-1} sucrose containing 50 mmol L^{-1} Tris-HCl, pH 7.5) with a Polytron. The homogenate was centrifuged at 4 °C. The supernatant was filtered through a single layer of nylon mesh and centrifuged at 40 000 *g* for 20 min at 4 °C. The resulting pellets were washed twice with ice-cold incubation buffer (50 mmol L^{-1} Tris-HCl, containing 10 mmol L^{-1} $MgCl_2$). The final pellet was resuspended with 3 to 10 volumes ice-cold incubation buffer and stored at −70 °C until use.

Preparation of C_2C_{12} cells was carried out using our previous method (Cheng & Liu 2000; Liu et al 2001). Briefly, culture medium of C_2C_{12} cells was replaced by 2 mL HEPES-buffered saline (pH 7.4) containing (in mmol L^{-1}): 137 NaCl, 5 KCl, 0.9 $MgSO_4$, 1.4 $CaCl_2$, 3 $NaHCO_3$, 0.6 Na_2HPO_4, 0.4 KH_2PO_4, 5.6 glucose and 20 HEPES. The cells were allowed to equilibrate for at least 12 min at 37 °C then they were used for the binding assay.

The radioligand binding assay was initiated by an addition of [^{3}H]prazosin at 10 nmol L^{-1} into the tube containing samples incubated with test compounds at indicated concentrations for 30 min. The incubation period was 1 h at 37 °C and this period was sufficient for equilibrium in the preliminary experiment. The incubation was terminated by addition of cold incubation HEPES. After passing through GF/B filters (Whatman) under vacuum filtration, samples were washed three times with 3 mL cold incubation HEPES. The samples in the filter were transferred into a liquid scintillation vial and 1 mL 0.5% Triton X-100/0.2 mol L^{-1} NaOH added. Liquid scintillation fluid (10 mL) was added and the samples were counted at 40% efficiency. Non-specific binding was defined as the bind-

ing obtained in the presence of 0.1 μmol L^{-1} prazosin. Assays were conducted in duplicate.

Chemicals

Cinnamic acid and analogues were obtained from Professor Feng-Lin Hsu (Taipei Medical University, Taipei City, Taiwan). Radioactive substances, 2-[^{14}C]deoxy-D-glucose and [^{3}H]prazosin, were the products of New England Nuclear (Boston, MA). Porcine insulin monocomponent was obtained from Novo Industrias (Bagsvaerd, Denmark). Cytochalasin B and others were purchased from Sigma Chemical Co. (St Louis, MO). DMEM and FBS were purchased from Gibco BRL (Gaithersburg, MD). All other reagents were from standard sources.

Data analysis

Parametric data were expressed as the mean ± s.e.m. The n in the text refers to the number of separate experiments. The concentration for production of 50% inhibition (IC50) was obtained from non-linear regression analysis. Multiple comparisons were analysed by analysis of variance and Dunnett's post-hoc test. The level of significance was uniformly set at $P < 0.05$.

Results and Discussion

To evaluate the α_1-adrenoceptor subtype, determination of the effect on glucose uptake into isolated tissue is useful (Cheng & Liu 2000). Cells in culture have proven to be appropriate for glucose transport studies because it seems better than the isolated preparations that have relatively short survival in-vitro (Sheriff et al 1992). Therefore, the mouse C_2C_{12} cell derived from the mouse skeletal muscle C_2 cell line was employed due to the similar properties of this cell line to isolated skeletal muscle. It has been indicated that α_{1A}-adrenoceptor is the major subtype of the α_1-adrenoceptors in the C_2C_{12} cell (Liu et al 2000). Thus, we have used these cells to investigate the effect of cinnamic acid analogues on α_{1A}-adrenoceptors.

The affinity of cinnamic acid and six analogues to α_{1A}-adrenoceptors was illustrated by radioligand binding experiment in C_2C_{12} cells. Figure 2 shows the displacement of [^{3}H]prazosin binding by these seven compounds in C_2C_{12} cells. Decrease of [^{3}H]prazosin binding was obtained in parallel with an increase of these analogues of cinnamic acid from 0.1 μmol L^{-1} to 5 mmol L^{-1} (Figure 2). As shown in Table 1, the IC50 value for cinnamic acid to displace [^{3}H]prazosin binding was only approximately 1.2 ± 0.7 μmol L^{-1}. According to Liu et al (2001), the IC50 value of isoferulic acid from the displacement of [^{3}H]prazosin binding was approximately 1.6 ± 0.4 μmol L^{-1}. Also, the IC50 value for 3,5-dimethoxy-4-hydroxycinnamic acid to displace [^{3}H]prazosin binding was approximately 3.1 ± 0.6 μmol L^{-1}. However, the IC50 value for ferulic acid and 2-hydroxycinnamic acid to displace [^{3}H]prazosin binding was approximately 82.3 ± 4.4 and 70.8 ± 3.1 μmol L^{-1}, respectively. The IC50 values of ferulic acid and 2-hydroxycinnamic acid were markedly higher than that of isoferulic acid, showing the lower affinity of the two compounds (Table 1).

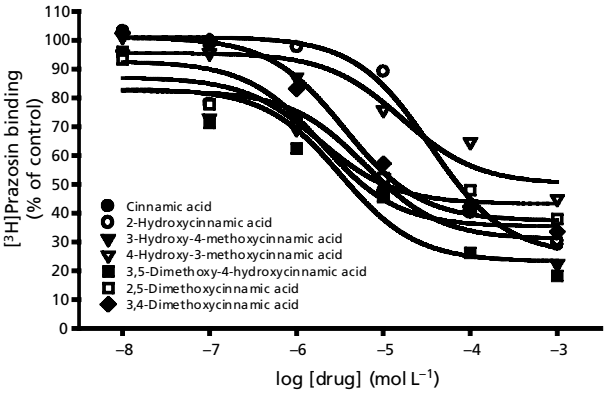


Figure 2 Displacement of [^{3}H]prazosin binding by cinnamic acid derivatives in C_2C_{12} cells. Each point represents the average of four experiments without the standard error bar of the mean.

Table 1 Inhibition of [^{3}H]prazosin binding to α_1-adrenoceptors in rat prostate.

Drug	n	IC50 (μmol L^{-1})
Cinnamic acid	4	1.2 ± 0.7
2-Hydroxycinnamic acid	4	70.8 ± 3.1
3-Hydroxy-4-methoxycinnamic acid	4	1.6 ± 0.4
4-Hydroxy-3-methoxycinnamic acid	4	82.3 ± 4.4
3,5-Dimethoxy-4-hydroxycinnamic acid	4	3.1 ± 0.6
2,5-Dimethoxycinnamic acid	4	1.8 ± 0.4
3,4-Dimethoxycinnamic acid	4	3.6 ± 0.8

Data shown are the mean ± s.e.m. The n refers to the number of experiments.

Similar to our previous reports (Cheng & Liu 2000; Liu et al 2001), incubation with caffeic acid (3,4-dihydroxycinnamic acid) or isoferulic acid (3-hydroxy-4-methoxycinnamic acid) enhanced the radioactive glucose uptake into C_2C_{12} cells in a concentration-dependent manner (Figure 3). Incubation with ferulic acid (4-hydroxy-3-methoxycinnamic acid) resulted in a smaller increase in 2-DG uptake into C_2C_{12} cells compared with isoferulic acid at the same concentrations. Moreover, stimulation of 2-DG uptake by 3,5-dimethoxy-4-hydroxycinnamic acid was more potent compared with isoferulic acid at 10 μmol L^{-1} but it was still markedly ($P < 0.05$) smaller compared with porcine insulin (1 nmol L^{-1}). Stimulation of 2-DG uptake into the C_2C_{12} cells after a 30-min exposure to 1 nmol L^{-1} porcine insulin was approximately 223.2 ± 3.3% (n = 6) of the basal 2-DG uptake that was taken as 100% from samples

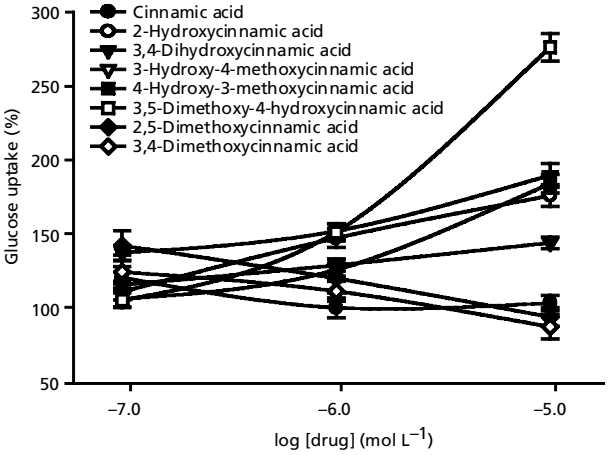


Figure 3 Effect of cinnamic acid derivatives on glucose uptake into C_2C_{12} cells. Results are the mean ± s.e.m. of seven determinations expressed as the percentage of the control that was obtained from C_2C_{12} cells incubated with DMEM without treatment.

without treatment. It seems that the hydroxyl group at the position of the third and/or fourth carbon on the benzene ring of cinnamic acid may raise the potency to enhance glucose uptake into C_2C_{12} cells. The analogue of cinnamic acid with the hydroxyl group at the second carbon on the benzene ring, 2-hydroxycinnamic acid, had the ability to enhance 2-DG uptake into C_2C_{12} cells at a level similar to that produced by isoferulic acid. However, the ability of cinnamic acid analogues to enhance glucose uptake into C_2C_{12} cells disappeared when the hydroxyl groups on the benzene ring of cinnamic acid were totally substituted for methoxyl groups. As shown in Figure 3, incubation with 2,5-dimethoxycinnamic acid or 3,4-dimethoxycinnamic acid diminished 2-DG uptake into C_2C_{12} cells gradually in a concentration-dependent manner. In addition to cinnamic acid, the 2-DG uptake induced by 2,5-dimethoxycinnamic acid or 3,4-dimethoxycinnamic acid at the highest concentration ($0.1\,\mu mol\,L^{-1}$) was significantly lower ($P < 0.05$) compared with the other compounds. Also, cinnamic acid itself without structural modification showed no effect on the glucose uptake into C_2C_{12} cells, indicating that cinnamic acid lacked the ability to activate α_{1A}-adrenoceptors. Otherwise, cinnamic acid and these two compounds displaced the specific binding of [^{3}H]prazosin in C_2C_{12} cells with small IC50 values. It has been indicated that the α_{1A}-adrenoceptor is the major subtype of α_1-adrenoceptors in C_2C_{12} cells (Liu et al 2000). Therefore, in addition to cinnamic acid, 2,5-dimethoxycinnamic acid or 3,4-dimethoxycinnamic acid can be considered as antagonists of α_{1A}-adrenoceptors. It has been established that the blockade of the α_{1A}-adrenoceptor is effective to relieve the symptoms of benign prostatic hypertrophy (Beduschi et al 1998). Application of two cinnamic acid analogues working as the antagonists of α_{1A}-adrenoceptors for the handling of benign prostatic hypertrophy requires further study. Nevertheless, increase of glucose uptake by cinnamic acid analogues seems to be related to the methoxylation and/or hydroxylation at the benzene ring of cinnamic acid. Therefore, the number and the position of a new group in cinnamic acid may play an important role in the stimulation of glucose uptake. Taken together, our data provide a positive relation between chemical modification of cinnamic acid via methoxylation and/or hydroxylation and the activation of α_{1A}-adrenoceptors, using the increase of glucose uptake into C_2C_{12} cells as the indicator.

It has been established that the dominant α_1-adrenoceptor subtype in rat prostate is the α_{1A}-adrenoceptor (Hiraoka et al 1999), while the α_{1B}-adrenoceptor subtype is predominantly found in the spleen of rat (Zhong & Minneman 1999). Usually, the selectivity of compounds for α_1-adrenoceptor subtypes has been assessed by the displacement of [^{3}H]prazosin binding in rat isolated prostate or spleen. The selectivity can be obtained from the ratio of the IC50 value in prostate and spleen for each compound (Zhong & Minneman 1999). Thus, we investigated four compounds (2-hydroxycinnamic acid, 3-hydroxy-4-methoxycinnamic acid, 3,5-dimethoxy-4-hydroxycinnamic acid, 4-hydroxy-3-methoxycinnamic acid) that possessed the ability to enhance glucose uptake into C_2C_{12} cells. In rat prostate, indicating the response of α_{1A}-adrenoceptors, decrease of [^{3}H]prazosin binding was obtained in parallel with an increase of the four cinnamic acid analogues from 0.01 to $500\,\mu mol\,L^{-1}$ (Figure 4). Figure 5 shows that [^{3}H]prazosin specific binding in rat spleen, indicating the response of α_{1B}-adrenoceptors, was decreased with an increase of these analogues from 0.01 to $500\,\mu mol\,L^{-1}$. The selectivity of the four cinnamic acid analogues between α_{1A}- and α_{1B}-adrenoceptors was evaluated. The IC50 values of isoferulic acid and 2-hydroxycinnamic acid to displace [^{3}H]prazosin binding in rat spleen were approximately 3- and 5-fold that to displace the [^{3}H]prazosin binding in rat prostate, respectively. Also, the IC50 value of ferulic acid to displace

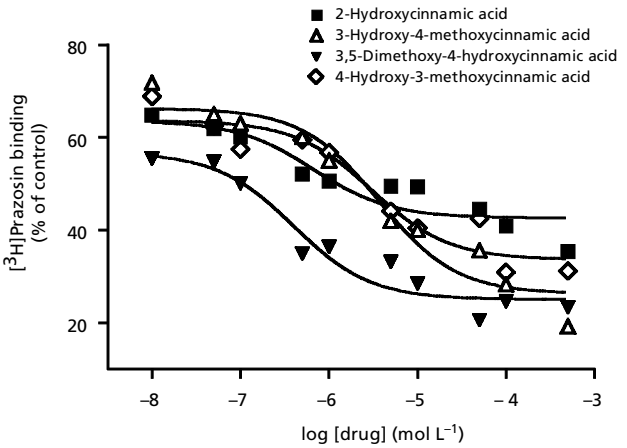


Figure 4 Displacement of [^{3}H]prazosin binding by cinnamic acid derivatives in rat prostate. Each point represents the average of four experiments without the standard error bar of the mean.

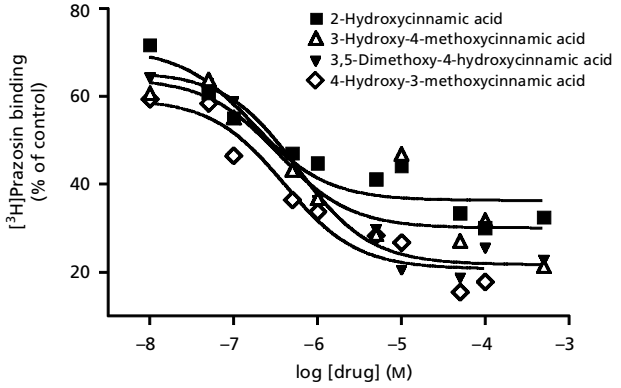


Figure 5 Displacement of [^{3}H]prazosin binding by cinnamic acid derivatives in rat spleen. Each point represents the average of four experiments without the standard error bar of the mean.

[^{3}H]prazosin binding in rat prostate and spleen was in the ratio of 1:200. However, the IC50 value of 3,5-dimethoxy-4-hydroxycinnamic acid to displace the [^{3}H]prazosin binding in rat prostate and spleen was only in the ratio of 1:3. Therefore, it seems that 3,5-dimethoxy-4-hydroxycinnamic acid had a similar affinity for α_{1A}- and α_{1B}-adrenoceptor subtypes. Taken together, it demonstrated that chemical modification to make the presence of a hydroxy group at the position of the fourth carbon on the benzene ring of cinnamic acid raised the affinity of compounds to bind with α_{1A}-adrenoceptor on rat prostate or C_2C_{12} cells. Furthermore, methoxylation near the fourth carbon on the benzene ring enhanced the potency of these cinnamic acid analogues to increase glucose uptake by activating the α_{1A}-adrenoceptor in C_2C_{12} cells. However, the relationships of these compounds between structures and the potency to enhance the glucose uptake remain to be defined.

Glucose transportation is the rate-limiting step in carbohydrate metabolism of skeletal muscle, a major site of glucose disposal (Baron et al 1998). Treatment of diabetes mellitus by insulin and/or oral drugs fails to prevent the complications associated with diabetes in many patients, indicating that additional treatment would be helpful. Previous studies suggested that agonists of α_{1A}-adrenoceptors would be valuable in the therapeutic control of diabetic subjects (Cheng & Liu 2000; Cheng et al 2000). Therefore, the results of this study are useful for the understanding of the structure–activity relationship between compounds in the basic structure of cinnamic acid that may help the development of new therapeutic adjuvants in the management of diabetes.

In conclusion, we have found four analogues of cinnamic acid that showed the ability to activate the α_{1A}-adrenoceptor subtype to enhance the glucose uptake into C_2C_{12} cells. These analogues were 2-hydroxycinnamic acid, isoferulic acid (3-hydroxy-4-methoxycinnamic acid), 3,5-dimethoxy-4-hydroxycinnamic acid and ferulic acid (4-hydroxy-3-methoxycinnamic acid).

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