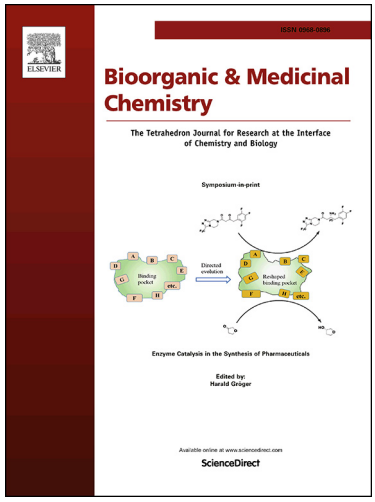
Accepted Manuscript



Synthesis of *N*-hydroxycinnamoyl amide derivatives and evaluation of their anti-oxidative and anti-tyrosinase activities

Dong Wang, Jun Zhu, Jin-Rong Xu, Dan-Dan Ji

PII: S0968-0896(19)30392-X
DOI: https://doi.org/10.1016/j.bmc.2019.05.031
Reference: BMC 14918

To appear in: *Bioorganic & Medicinal Chemistry*

Received Date: 8 March 2019
Revised Date: 15 May 2019
Accepted Date: 19 May 2019

Please cite this article as: Wang, D., Zhu, J., Xu, J-R., Ji, D-D., Synthesis of *N*-hydroxycinnamoyl amide derivatives and evaluation of their anti-oxidative and anti-tyrosinase activities, *Bioorganic & Medicinal Chemistry* (2019), doi: https://doi.org/10.1016/j.bmc.2019.05.031

Synthesis of *N*-hydroxycinnamoyl amide derivatives and evaluation of their anti-oxidative and anti-tyrosinase activities

Dong Wang[a], Jun Zhu[a,b,*] Jin-Rong Xu[a], Dan-Dan Ji[a]

[a] *School of Science, Beijing Technology and Business University, Beijing 100048, China*

[b] *Key Laboratory of Cosmeyic, China National Light Industry, Beijing 100048, China*

[*] *Corresponding author: E-mail address: zhujun@btbu.edu.cn (J. Zhu)*

Abstract

Twelve *N*-hydroxycinnamoyl amino acid amide ethyl esters (CAES) were synthesized by using L-amino acid ethyl ester hydrochloride and corresponding cinnamic acid (ferulic acid, acetylferulic acid and caffeic acid) as raw materials in the presence of a catalytic amount of 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide-hydrochloride (EDC) and 1-hydroxybenzotriene (HOBt). The 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radical scavenging activities of CAES were evaluated. The anti-tyrosinase activities of *N*-feruloyl amino acid ethyl esters and the hydroxyl (OH·) free radical scavenging activities of *N*-caffeoyl amino acid ethyl esters were also examined. DPPH free radical scavenging activity was shown in all CAES, of which *N*-caffeoyl amino acid ethyl esters demonstrated higher radical scavenging activity than *N*-feruloyl amide derivatives, and (E) -*N*-(caffeic acid)-L-glycinate ethyl ester (**c**$_5$) had the strongest ability to scavenge free radicals with an IC_{50} value of 18.6 μM.. The acetylferuloyl amino acid esters exhibited the highest tyrosinase inhibition activity among the tested amides.

Keywords: *N*-hydroxycinnamoyl amino acid amide ethyl esters;L-amino acid ethyl ester hydrochloride;Radical scavenging; Anti-tyrosinase activit

1. Introduction

The pigmentation of plants and animals is usually triggered by enzymatic browning, mainly caused by tyrosainase catalysis.[1] The pigmentation of melanin appears in food, causing the undesirable reduction of nutritional quality and consumer acceptance, and its commercial value may also be affected. The browning degree of fruits and vegetables is mainly influenced by the content of phenols and the activity of tyrosinase.[2] Moreover, pigmentation-related disorders of human might be caused by melanin, such as melasma and postinflammatory melanoderma.[3] Tyrosinase is the main rate-limiting enzyme in melanin synthesis. In addition, experimental evidence suggested that the excess free radicals in the body are one of the important factors that exacerbate melanin production.[4] Free radicals are produced continuously during normal physiological events, which not only lead to the accumulation of lipid peroxides, but also exacerbate cellular injury and aging process.[5-7] However, antioxidant defense mechanisms reduce the production of free radicals. Therefore, exploration for high-efficiency tyrosinase inhibitors and anti-oxidants is important for food preservation and medicinal whitening products.

Caffeic acids (CA) and Ferulic acids (FA) as hydroxycinnamic acid derivatives,[8] are widely distributed in fruits, vegetables and drinks.[9-12] Natural occurring CA and FA are very unstable in structure, they are usually presented as conjugated (esters and amides) forms.[13] Hydroxycinnamamide is one important kind of hydroxycinnamic acid derivatives and is also widely distributed in plants.[14] The plant hydroxycinnamamides, exhibited a wide variety of biological activities, including anti-tyrosinase,[8,15] anti-viral,[16] anti-oxidative,[16-18] anti-inflammatory[19-21] and insulin secretion stimulating activities in *vitro*.[22]

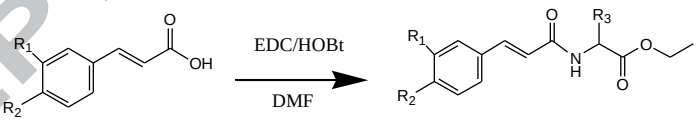
Interestingly, hydroxycinnamic acid amide derivatives formed by the hydroxycinnamic acid with amino acids had a significantly stronger antioxidant activity than hydroxycinnamic acid, and the

irritation were significantly reduced.[5,23] Moreover, a large number of experimental datas indicated that tyrosinase inhibitors have an enhanced inhibitory effect on tyrosinase as their hydrocarbon chain length increases.[1,24]

Inspired by the above studies, twelve *N*-hydroxycinnamoyl amino acid amide ethyl esters (CAES) (their structure was shown in **Table 1**) were synthesized by using L-amino acid ethyl ester hydrochloride and cinnamic acid (ferulic acid, acetylferulic acid and caffeic acid) as raw materials and the structure-activity relationship (antioxidative and anti-tyrosinase activity) was evaluated between *N*-hydroxycinnamic acid and L-amino acid ethyl ester. In this study, the anti-oxidative activity of CAES was evaluated by DPPH free radical scavenging rate. In addition, the anti-tyrosinase of *N*-feruloyl amides and the hydroxyl free radical scavenging activities of *N*-caffeoyl amides were studied.

Table 1

Synthesized *N*-hydroxycinnamoyl amino acid amides ethyl esters (CAES)



d_1～d_3 a_1～a_3, b_1～b_3, c_1～c_6

Compound	R_1	R_2	R_3	Yields, %
a_1	OCH_3	OH	CH_3	48.4
a_2	OCH_3	OH	$CH(CH_3)_2$	50.6
a_3	OCH_3	OH	$CH_2CH(CH_3)_2$	55.8
b_1	OCH_3	$OOCCH_3$	CH_3	54.8

b_2	OCH_3	$OOCCH_3$	$CH(CH_3)_2$	52.7
b_3	OCH_3	$OOCCH_3$	$CH_2CH(CH_3)_2$	54.8
c_1	OH	OH	CH_3	73
c_2	OH	OH	$CH(CH_3)_2$	79
c_3	OH	OH	$CH_2CH(CH_3)_2$	82
c_4	OH	OH	$CH(CH_3)CH_2CH_3$	86
c_5	OH	OH	H	60
c_6	OH	OH	$CH_2C_6H_6$	75
CA(d_1)	OH	OH	–	–
FA(d_2)	OCH_3	OH	–	–
d_3	OCH_3	$OOCCH_3$	–	71.4

2. Results and discussion

2.1 Biological evaluation

2.1.1. DPPH radical scavenging activity

DPPH had an unpaired valence electron at one atom of nitrogen bridge, when combined with a hydrogen-donating antioxidant, the power of free radical scavenging of an antioxidant can be determined by measuring of a color change at 517 nm.[25] The inhibitory effects of the synthesized amides (see **Table 1.** for structures) against stable free DPPH radical (IC_{50}) were shown in **Table 2.** Ferulic acid, caffeic acid and ascorbic acid were tested as controls. Their DPPH radical scavenging activity

among the tested target products decreased in the following order: **c_5** > **c_2** > **CA (d_1)** > **c_3** > **c_1** > **c_6** > **FA (d_2)** > **c_4** > **a_1** > **a_3** > **a_2** > Vitamin C > **b_1** > **d_3**, **b_2**, **b_3**. All CAES exhibited inhibitory effects on free DPPH radicals. As shown in **Table 2.** *N*-caffeoyl amino acid ethyl esters demonstrated higher radical scavenging activity than *N*-feruloyl amide derivatives. This results suggests that the introduction of (hydroxyl) groups significantly increases the scavenging free radical activity of these compounds. Furthermore, the scavenging effect of the same parent molecule demonstrated the following sequence: ferulic acid (FA) > feruloyl amino acid ethyl esters > acetylferuloyl amino acid ethyl esters, with feruloyl amino acid esters exhibited higher scavenging activity than acetylferuloyl amino acid esters with phenolic hydroxyl groups. As for the IC_{50} values, *N*-caffeoyl amide derivatives showed the order of **c_5** > **c_2** > **CA (d_1)**. It is noticeable that *N*-caffeoyl amino acid ethyl esters are more effective than ascorbic acid in DPPH-scavenging activity. Among all *N*-caffeoyl amino acid ethyl esters, (E) -*N*-(caffeic acid)-L-glycinate ethyl ester (**c_5**) showed the strongest ability to scavenge free radicals, with an IC_{50} of 18.6 μM. Correspondingly, for the series *N*-feruloyl amides derivatives had no enhancing effect on DPPH inhibition in the DPPH-scavenging test. The activity of different CAES correlates with their alkyl chains.

Table 2

DPPH radical scavenging activity

Compound	IC_{50}（μM）	Compounds	IC_{50}（μM）
d_3	>500	**c_1**	29.7

a_1	46.4	**c_2**	19.6
a_2	59.6	**c_3**	28.6
a_3	56.1	**c_4**	43.5
b_1	482	**c_5**	18.6
b_2	>500	**c_6**	33.2
b_3	>500	**Vitamin C**	80.9
FA(d_2)	35.7	**CA(d_1)**	27.6

Note: Ferulic acid, caffeic acid and Vitamin C were tested as positive controls. Each experiment was measured for 3 times. The IC_{50} value was defined as the concentration of the antioxidant required when reaching a 50% inhibition of DPPH free radicals. The IC_{50} value was determined by interpolation,from linear regression analysis.

2.1.2. Hydroxyl radical scavenging activity of *N*-caffeoyl amino acid ethyl esters

In this study, *N*-caffeoyl amino acid ethyl esters demonstrated higher DPPH radical scavenging activity than *N*-feruloyl amides derivatives. Oxidation is one of the important causes of food deterioration. H_2O_2 as a nonradical that belongs to the group of ROS.[6] can cause serious damage to biomolecules via the formation of OH·. Hydroxyl radicals are commonly formed *in vivo* and continuously produced during normal physiologic events. Hydroxyl radicals usually cause damage to lipids, proteins, and nucleic acids.[26]

The effect of *N*-caffeoyl amino acid ethyl esters in hydroxyl radical-scavenging activity was measured *in vitro* and the obtained results (IC_{50}) were shown in **Table 3**. The scavenging activity of *N*-caffeoyl amino acid esters on OH· radicals decreased in the following order: **c_1 > CA (d_1) > c_6 > c_5 > c_2 > c_3 > c_4**

> Vitamin C. It is noticable that *N*-caffeoyl amino acid ethyl esters showed higher anti-oxidative effect than ascorbic acid (a positive control). Fan reported that the antioxidative activity of (E) -*N*-(caffeic acid)-L-phenylalaninate ethyl ester is lower than the free CA.[24] The present work found that (E) -*N*-(caffeic acid) -L-alanine ethyl ester (**c_1**) demonstrated higher hydroxyl radical scavenging activity than (E) -*N*-(caffeic acid)-L-phenylalaninate ethyl ester (**c_6**) and the free **CA (d_1)**.

Table 3

Hydroxyl radical-scavenging activity

Compounds	IC_{50}（mM）	Compounds	IC_{50}（mM）
c_1	0.214	**c_5**	0.340
c_2	0.348	**c_6**	0.292
c_3	0.373	**CA(d_1)**	0.287
c_4	0.411	Vitamin C	1.616

Note: Vitamin C were tested as positive controls. Each experiment was measured for 3 times. IC_{50} (sample concentration in mM at which the hydroxyl radical inhibition rate reaches 50%). The IC_{50} value was determined by interpolation, from linear regression analysis.

2.1.3. Anti-tyrosinase activity

Gómezcordovés reported that caffeic acid and ferulic acid in wine showed inhibition of tyrosinase activity.[27] (E) -*N*-(feruloyl acid)-L-phenylalaninate ethyl ester (IC_{50} < 0.18 μM) demonstrated higher inhibition of tyrosinase activity than (E) -*N*-(caffeic acid)-L-phenylalaninate ethyl ester (IC_{50} > 29 μM).[15] Considering feruloyl amino acid esters and their acetylferuloyl amino acid esters derivatives are potential inhibitors, the effects of the tested target products in suppressing mushroom tyrosinase activity

was measured and the obtained results were summarized in **Fig.1**. The inhibitory rate decreased in the following order: **b_3** > **b_2** > **b_1** > **a_1** > **FA (d_2)** > **a_3** > **d_3** > **a_2**. It was found that acetylferuloyl amino acid amide and feruloyl amino acid amide, (E) -ethyl 2-(3-(4-acetoxy-3-methoxyphenyl)acrylamido) -4-methylpentanoate (**b_3**) showed the most significant inhibitory effects agaist mushroom tyrosinase than ferulic acid ($p < 0.05$). Furthermore, the anti-tyrosinase activity of *N*-feruloyl amides derivatives had the following sequence: acetylferuloyl amino acid esters > feruloyl amino acid esters and ferulic acid (FA). The preliminary results of the structure-activity relationship were as follows: 1) the tyrosinase inhibition activity of acetylferuloyl amino acid esters was more effective than feruloyl amino acid amides, which mainly derive from the acetyl groups of the molecular structure; 2) The anti-tyrosinase activity of different feruloyl amino acid esters and their acetylferuloyl amino acid ester derivatives was found to correlate with the alkyl chains in structure.

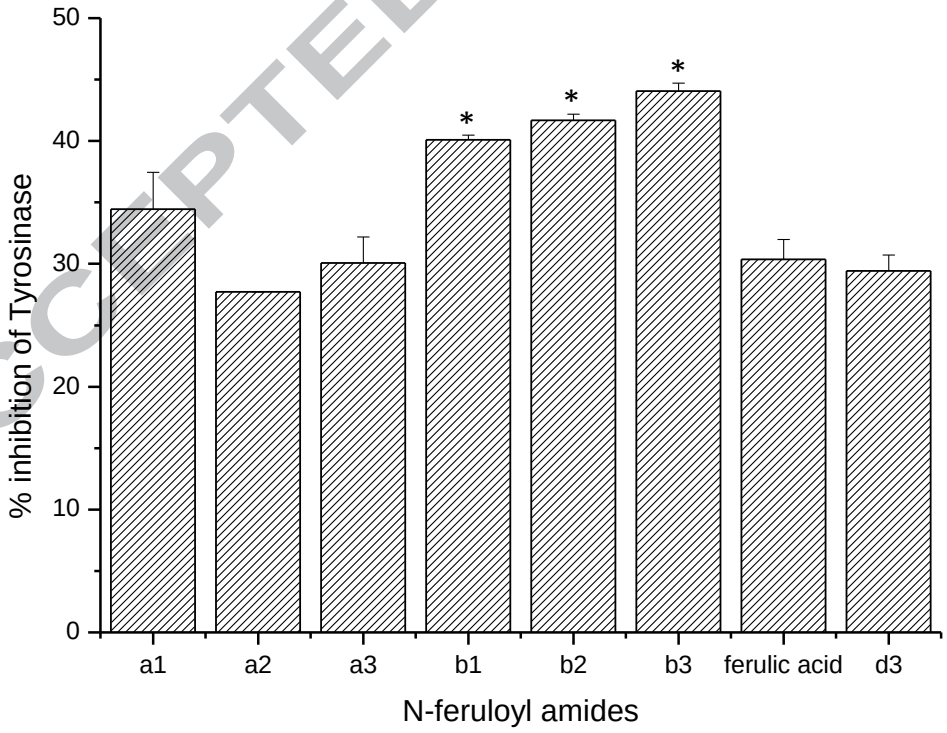


Fig.1.

Anti-tyrosinase activity of different CAES derivatives (**a_1- a_3**, **b_1- b_3** and **d_3**). The concentrations of the

CAES derivatives are 1 mg/L. The values were presented as mean ± confidence interval (n=3). * P < 0.05 (Compared with the ferulic acid group, calculated at level of significance 0.05).

3. Conclusions

Twelve CAES have been synthesized and their efficacy as anti-oxidant was measured by acavenger activity against DPPH radicals. In addition, the anti-tyrosinase of *N*-feruloyl amides and the OH· scavenging-activities of *N*-caffeoyl amides were studied.

The present study has shown that *N*-caffeoyl amino acid ethyl esters exhibited the highest antioxidant activity in scavenging both DPPH· and OH· radicals when compared with Vitamin C (a famous antioxidant). The radical-scavenging activity of amides mainly depended on the phenolic hydroxyl groups, carboxyl groups and alkyl chain of the molecular structure. On the other side, acetylferuloyl amino acid esters exhibited the highest tyrosinase inhibition activity among the tested amides. Further investigations are required to elucidate the relationship between the activity and alkyl chain structure. All the data suggested that N-caffeoyl amino acid ethyl esters might serve as new potent anti-oxidative and acetylferuloyl amino acid esters might serve as new whitening agents used in food or cosmetics industry.

4. Experimental section

4.1. Chemistry

Ferulic acid, Caffeic acids were purchased from Macklin Biochemical (Shanghai) and DPPH was purchased from TCI (Shanghai). Mushroom tyrosinase and L-ascorbic acid was purchased from Beijing Huabo Station Bioanalysis Technology Co., Ltd., China. All other chemical reagents were in analytical grade.

The NMR spectra of the synthesized compounds were acquired on a Bruker AV 300 MHz spectrometers, operating at 300 MHz for ^1H (the synthesized compounds were dissolved in $CDCl_3$ or DMSO-d_6); The model of UPLC-MS was ACQUITY UPLC/Xevo G2. The reduction in absorbance of DPPH and anti-tyrosinase assays were measure by ELISA (Tecan, infinite M_{200} PRO, Swiss).

4.2. Preparation of acetylferulic acid (d_3)

Ferulic acid (3.88 g, 0.02 mol) was dissolved in acetic anhydride (10 mL) with the addition of anhydrous pyridine (10 drops). After that, the resultant reaction mixture was heating back for 7 h at 120 ℃. The mixture was cooled at 25 ℃ with the addition of water (40 mL), and then the reaction mixture was stirred for 2 h until the appearance of white precipitate at 25 ℃. Finally, the white precipitate was filtered, and the residual paste was collected and purified by EtOH.

4.3. Preparation of CAES

Feruloyl amino acid ethyl esters **a_{1-3}**, **b_{1-3}** were synthesized from ferulic acid and different amino acid ethyl esters using 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide-hydrochloride (EDC) /1-hydroxybenzotriene (HOBt) method with some modifications.[28]

Ferulic acid (1.07 g, 5.5 mmol) and HOBt (0.74 g, 5.5 mmol) and EDC（0.99 g, 5.5 mmol） were dissolved in (11 mL) *N*,*N*-dimethylformamide (DMF), the reaction mixture was cooled at 0 ℃ and reacted for 10 min. Then, *N*-methyl morpholine (0.76 mL) and hydrochloride of HCl•NH_2-CH(R)-COOR (C-protected amino acid) (5.5 mmol) in 8.5 ml CH_2Cl_2 were added and well mixed. The reaction was carried out at 0 ℃ for 1 h, followed by stirring at room temperature for 20 h. When the reaction was completed, the reaction solution was poured into (100 mL) 5% $NaHCO_3$ and extracted with ethyl acetate (2 × 75 mL). Then, the organic phase was washed with water (2 × 50 mL) and brine (2 × 50 mL) before dring with anhydrous Na_2SO_4 for 24 h. After recovering the solvent under reduced pressure, the residue

was purified by silica gel column chromatography (Ethyl acetate/Petroleum ether (2:1)) to give **a_1−a_3**. The preparation of acetylferuloyl amino acid ethyl esters **b_1−b_3** was the same as feruloyl amino acid esters' except for the use acetylferulic acid as reaction material instead of ferulic acid. EDC/HOBt coupling method for the synthesis of caffeoyl amino acid ethyl esters **c_1−c_6** was applied.[5]

(E) -3-(4-acetoxy-3-methoxyphenyl) acrylic acid (***d_3***): yield 71.4%, white powder, melting point: 193-195 ℃; ^{1}H NMR (300 MHz, $CDCl_3$): 7.74 (d, *J* = 15.6 Hz, 1H, CH=C), 7.06-7.17 (m, 3H, Ar-H), 6.40 (d, *J* = 15.6 Hz, 1H, C=CH), 3.88 (s, 3H, OCH_3), 2.33 (s, 3H, $OCOCH_3$); ESI-MS (m/*z*): 259.0591 ([M+Na]+).

(E) -N-(feruloyl) -L-alanine ethyl ester (***a_1***): yield 48.4%, white powder, melting point: 110-112 ℃; ^{1}H NMR (300 MHz, $CDCl_3$): 7.53 (d, *J* = 15.6 Hz, 1H, CH=C), 7.53 (dd, *J* = 8.1, 1.8 Hz, 1H, Ar-H), 6.96 (d, *J* = 1.8 Hz, 1H, Ar-H), 6.88 (d, *J* = 8.1 Hz, 1H, Ar-H), 6.36 (d, *J* = 7.2 Hz, 1H, -NH), 6.30 (d, *J* = 15.6 Hz, 1H, C=CH), 6.10 (s, 1H, -OH), 4.72 (m, 1H, -CH), 4.22 (q, *J* = 7.2 Hz, 2H, $-CH_2$), 3.89 (s, 3H, $-OCH_3$), 1.46 (d, *J* = 7.2 Hz, 3H, $-CH_3$), 1.29 (t, *J* = 7.2 Hz, 3H, $-CH_3$); ESI-MS (m/*z*): 294.1344 ([M+H]+).

(E) -N-(feruloyl) -L-valine ethyl ester (***a_2***): yield 50.6%, white powder, melting point: 178-180 ℃; ^{1}H NMR (300 MHz, $CDCl_3$): 7.55 (d, *J* = 15.6 Hz, 1H, CH=C), 7.04 (dd, *J* = 8.4, 1.8 Hz, 1H, Ar-H), 6.99 (d, *J* = 1.8 Hz, 1H, Ar-H), 6.91 (d, *J* = 8.4 Hz, 1H, Ar-H), 6.34 (d, *J* = 15.6 Hz, 1H, C=CH), 6.24 (d, *J* = 8.7 Hz, 1H, -NH), 6.13 (s, 1H, -OH), 4.72 (dd, *J* = 8.7, 4.8 Hz, 1H, -CH), 4.23 (dddd, *J* = 21.6, 14.4, 7.2, 2.4 Hz, 2H, $-CH_2$), 3.90 (s, 3H, $-OCH_3$), 2.23 (m, 1H, -CH), 1.30 (t, *J* = 7.2 Hz, 3H, $-CH_3$), 0.97 (dd, *J* = 9.9, 6.9 Hz, 6H, $>CH_3+CH_3$); ESI-MS (m/*z*): 322.1662 ([M+H]+).

(E) -N-(feruloyl) -L-leucine ethyl ester (***a_3***): yield 55.8%, white powder, melting point: 180-182 ℃; ^{1}H NMR (300 MHz, $CDCl_3$): 7.53 (d, *J* = 15.6 Hz, 1H, CH=C), 7.02 (dd, *J* = 8.1, 1.8 Hz, 1H, Ar-H),

6.96 (d, *J* = 1.8 Hz, 1H, Ar-H), 6.88 (d, *J* = 8.1 Hz, 1H, Ar-H), 6.27 (d, *J* = 15.6 Hz, 1H, C=CH), 6.15 (d, *J* = 8.4 Hz, 1H, -NH), 5.97 (s, 1H, -OH), 4.78 (m, 1H, -CH), 4.21 (q, *J* = 7.2 Hz, 2H, $-CH_2$), 3.90 (s, 3H, $-OCH_3$), 1.56-1.72 (m, 3H, $>CH_2$+CH), 1.29 (t, *J* = 7.2 Hz, 3H, $-CH_3$), 0.96 (t, *J* = 6.6 Hz, 6H, $>CH_3+CH_3$); ESI-MS (m/z): 336.1815 ([M+H]+).

(E) -ethyl 2-(3-(4-acetoxy-3-methoxyphenyl) acrylamido) propanoate (***b₁***): yield 54.8%, white solid, melting point: 80-82 ℃; ^{1}H NMR (300 MHz, $CDCl_3$): 7.57 (d, *J* = 15.6 Hz, 1H, CH=C), 7.11 (dd, *J* = 8.1, 1.8 Hz, 1H, Ar-H), 7.06 (d, *J* = 1.8 Hz, 1H, Ar-H), 7.02 (d, *J* = 8.1 Hz, 1H, Ar-H), 6.38 (d, *J* = 15.6 Hz, 1H, C=CH), 6.34 (d, *J* = 7.2 Hz, 1H, -NH), 4.72 (m, 1H, -CH), 4.23 (q, *J* = 7.2 Hz, 2H, $-CH_2$), 3.85 (s, 3H, $-OCH_3$), 2.32 (s, 3H, $-OCOCH_3$), 1.47 (d, *J* = 6.9 Hz, $-CH_3$), 1.30 (t, 3H, *J* = 7.2 Hz, $-CH_3$); ESI-MS (m/z): 336.1456 ([M+H]+).

(E) -ethyl 2-(3-(4-acetoxy-3-methoxyphenyl)acrylamido) -3-methylbutanoate (***b₂***): yield 52.7%, white solid, melting point: 100-102 ℃; ^{1}H NMR (300 MHz, $CDCl_3$): 7.57 (d, *J* = 15.6 Hz, 1H, CH=C), 7.09 (dd, *J* = 8.1, 1.8 Hz, 1H, Ar-H), 7.07 (d, *J* = 1.8 Hz, 1H, Ar-H), 7.02 (d, *J* = 8.1 Hz, 1H, Ar-H), 6.41 (d, *J* = 15.6 Hz, 1H, C=CH), 6.25 (d, *J* = 9 Hz, 1H, -NH), 4.70 (dd, *J* = 9, 4.8 Hz, 1H, -CH), 4.22 (dddd, *J* = 21.3, 14.4, 7.2, 1.8 Hz, 2H, $-CH_2$), 3.85 (s, 3H, $-OCH_3$), 2.31 (s, 3H, $-OCOCH_3$), 2.24 (m, 1H, -CH), 1.30 (t, 3H, *J* = 7.2 Hz, $-CH_3$), 0.97 (dd, *J* = 9.9, 6.9 Hz, 6H, $>CH_3+CH_3$); ESI-MS (m/z): 364.1770 ([M+H]+).

(E) -ethyl 2-(3-(4-acetoxy-3-methoxyphenyl)acrylamido) -4-methylpentanoate (***b₃***): yield 54.8%, yellow solid, melting point: 102-104 ℃; ^{1}H NMR (300 MHz, $CDCl_3$): 7.51 (d, *J* = 15.6 Hz, 1H, CH=C), 6.97-7.03 (m, 3H, Ar-H), 6.43 (d, *J* = 8.4 Hz, 1H, -NH), 6.34 (d, *J* = 15.6 Hz, 1H, CH=C), 4.76 (m, 1H, -CH), 4.20 (q, *J* = 7.2 Hz, 2H, $-CH_2$), 3.82 (s, 3H, $-OCH_3$), 2.30 (s, 3H, $-OCOCH_3$), 1.55-1.70 (m, 3H, $>CH_2$+CH), 1.28 (t, 3H, *J* = 7.2 Hz, $-CH_3$), 0.95 (t, *J* = 6.3 Hz, 6H, $>CH_3+CH_3$); ESI-MS (m/z): 378.1923 ([M+H]+).

(E) -N-(caffeic acid) -L-isoleucine ethyl ester (***c₄***): yield 86%, yellow solid, melting point: 120-124 ℃ ; ^{1}H NMR (300 MHz, DMSO-d_6): 9.36 (s, 1H, OH), 9.12 (s, 1H, OH), 8.19 (d, *J* = 8.2 Hz, 1H, NH), 7.23 (d, *J* = 15.7 Hz, 1H, HC=C), 6.93 (d, *J* = 1.7 Hz, 1H, Ar-H), 6.83 (d, *J* = 8.2 Hz, 1H, Ar-H), 6.73 (d, *J* = 8.1 Hz, 1H, Ar-H), 6.51 (d, *J* = 15.7 Hz, 1H, C=CH), 4.35-4.25 (m, 1H, CH), 4.10 (dd, *J* = 7.0, 5.1 Hz, 2H, OCH_2), 1.88-1.73 (m, 1H, CH), 1.51-1.04 (m, 5H, CH_2+CH_3), 0.92-0.80 (m, 6H, CH_3+CH_3); ESI-MS (m/*z*): 322.0833[M+H]+).

(E) -N-(caffeic acid)-L-phenylalaninate ethyl ester (***c₆***): yield 75%, yellow solid, melting point: 164-166 ℃; ^{1}H NMR (300 MHz, DMSO-d_6): 9.37 (s, 1H, OH） , 9.13 (s, 1H, OH), 8.42 (d, *J* = 10.2 Hz, 1H, NH), 7.23 (m, *J* = 15.9 Hz, 7.9 Hz, 5H, HC=C, Ar-H), 6.92 (d, *J* = 1.6 Hz, 1H, Ar-H), 6.81 (s, 1H, Ar-H), 6.73 (d, *J* = 8.2 Hz, 1H, Ar-H), 6.37 (d, *J* = 15.7 Hz, 1H, C=CH), 4.57-4.49 (m, 1H, CH), 3.09-2.81 (m, 2H, OCH_2), 1.10 (t, *J* = 7.1 Hz, 3H, CH_3); ESI+-MS (m/*z*): 356.0579 [M+H]+).

Detailed characterization data of the compounds (E) -N-(caffeic acid) -L-alanine ethyl ester (c1), (E) -N-(caffeic acid) -L-valine ethyl ester (c2), (E) -N-(caffeic acid) -L-leucine ethyl ester (***c₃***) and (E) -N-(caffeic acid)-L-glycinate ethyl ester (***c₅***) were showned in our previous paper.[23,29]

4.4. DPPH free radical scavenging activity

The free radical scavenging activity of the CAES was determined by the DPPH , and the activity was measured according to a previous literature, with minor modifications.[30] Testing samples were dissolved in absolute ethanol and formulated into different concentrations. Then, 1 mL of the sample solution was mixed with 1 mL of DPPH (0.2 mM) in ethanol with gentle shaking, the mixture was incubated in the dark for 30 min, and the absorbance at 517 nm was measured against blank control. 1 mL of absolute ethanol was used instead of the sample as the blank control. The positive control was L-ascorbic acid. Each experiment was measured for 3 times. For each sample ,an IC_{50} values was calculated using a dose-response curve. The IC_{50} value was defined as the concentration of the antioxidant required when

reaching an inhibition rate of 50%. Sample identified with a lower IC_{50} indicates higher anti-oxidative activity.[31]

4.5. The hydroxyl free radical-scavenging activity test

The hydroxyl free radical-scavenging activity of caffeoyl amino acid ethyl esters and L-ascorbic acid was determined according to the reported method with some modifications.[15] Briefly, 1mL of ethanolic solution of phenanthroline (0.75 mM), phosphate buffer (0.2 M, pH 7.4), sample solution (dissolved in DMSO) and aqueous solution of 0.75 mM $FeSO_4$ was mixed in a test tube with shaking. 1 mL of 0.01% H_2O_2 solution (30% H_2O_2 were mixed with deionised water) were added to the tube. Then the reaction mixed solution was incubated at 37 ℃ in a water bath for 1h, and the absorbance value of the mixture was measured at 536 nm in a spectrophotometer. Each experiment was measured for 3 times. The inhibition percentage of hydroxyl radical of the samples was expressed as IC_{50} (sample concentration in mM at which the hydroxyl radical inhibition rate reaches 50%). The inhibition percentage of OH· was determined using the following equation:

Hydroxyl radical inhibition rate (%) = [(C-B) / (A-B)] × 100

where A is the absorbance of the control, B is the initial concentration of the blank and C is the absorbance in the presence of sample

4.6. Assay of anti-tyrosinase activity

Anti-tyrosinase activity was determined according to the modified method using L-tyrosine as substrate.[8] Mushroom tyrosinase (25 KU/mg In a 3 ml reaction mixture containing L-ascorbic acid (pH 6.5, 25 ° C), ΔA265 changes to 0.001 per unit of enzyme activity unit per minute). Briefly, the compounds (1 g/L) (**a_1- a_3, b_1- b_3 , d_3** and **ferulic acid**) were dissolved in dimethyl sulfoxide (DMSO). HCl (0.1 M) and Phosphate buffer (0.2 M, pH 6.8) were dissolved in deionized water. The type and amount of reagents were shown in **Table 4**.

Table 4

The type and amount of reagents

tube	PB（mL）	L-tyrosine（mL）	sample（mL）	DMSO（mL）	MT（mL）
A	1.35	1.00	-	0.35	0.30
B	1.65	1.00	-	0.35	-
C	1.35	1.00	0.35	-	0.30
D	1.65	1.00	0.35	-	-

Note: Phosphate buffer (PB) (65 mL, 0.2 M, pH 6.8); Tyrosine (0.3 mL, 0.507 g/L) was dissolved in HCl (35 mL, 0.1 M) and phosphate buffer (65 mL, 0.2 M, pH 6.8); Inhibitor (0.350 mL, 1 g/L) was dissolved in DMSO; Mushroom tyrosinase (MT) (0.300 mL, 200 U/mL) dissolved in phosphate buffer (0.2 M, pH 6.8). The reference solution was obtained by replacing the inhibitor with DMSO (0.350 mL).

The reaction solution was incubated after adding mushroom tyrosinase solution to the mixture. Then, the mixture was incubated at 37 ℃ for 30 min. The absorbance was measured at 475 nm. The inhibition percentage of mushroom tyrosinase activity was determined by using the following formula:

% Tyrosinase activity inhibition rate = {[(A-B)-(C-D)]/(A-B)}×100%

Each experiment was measured for 3 times. Ferulic acid was used as positive control.

4.7. Statistical analysis

The IC_{50} values of DPPH and OH· free radical scavenging assay were obtained by regression analysis. The other data were presented as mean ± standard deviations and analyzed by SPSS software. The set of data was considered to have a significant difference ($p < 0.05$).

Acknowledgements

This work was financially supported by Outstanding Teachers' Reword of Beijing Colleges and Universities, China (No. 19005753005).

References

[1] Huang X H, Chen Q X, Wang Q, et al. Inhibition of the activity of mushroom tyrosinase by alkylbenzoic acids [J]. Food Chemistry, 2006, 94(1): 1-6

[2] Martinez M V, Whitaker J R. The biochemistry and control of enzymatic browning [J]. Trends Food Sci Technol, 995, 6(6): 195-200.

[3] Sang J L, Hann S K, Im S. The pigmentary system: physiology and pathophysiology, second edition [J]. Tidsskrift for Den Norske Legeforening, 2015, 86(9): 618-621.

[4] Stefania B, Emanuela C, Mauro P. Chemical and instrumental approaches to treat hyperpigmentation [J]. Pigment Cell & Melanoma Research, 2010, 16(2): 101-110.

[5] Wei Q Y, Jiang H, Zhang J X, et al. Synthesis of *N* -hydroxycinnamoyl amino acid ester analogues and their free radical scavenging and antioxidative activities [J]. Medicinal Chemistry Research, 2012, 21(8): 1905-1911.

[6] Gulcin I. Antioxidant activity of caffeic acid (3, 4-dihydroxycinnamic acid) [J]. Toxicology, 2006, 217(2-3): 213-220.

[7] Cheng J C, Fang J G, Chen W F, et al. Structure–activity relationship studies of resveratrol and its analogues by the reaction kinetics of low density lipoprotein peroxidation [J]. Bioorganic Chemistry, 2006, 34(3): 142-157.

[8] Chochkova M, Stoykova B, Ivanova G, et al. *N* -Hydroxycinnamoyl amides of fluorinated amino acids: synthesis, anti-tyrosinase and DPPH scavenging activities [J]. Journal of Fluorine Chemistry, 2013, 156(12): 203-208.

[9] Zhao Z, Moghadasian M H. Chemistry, natural sources, dietary intake and pharmacokinetic properties of ferulic acid: A review [J]. Food Chemistry, 2008, 109(4): 691-702.

[10] Mattila P, Pihlava J M, Hellstr ¶M J. Contents of phenolic acids, alkyl- and alkenylresorcinols, and avenanthramides in commercial grain products [J]. J Agric Food Chem, 2005, 53(21): 8290-8295.

[11] Mattila P, Hellstr M J, Törrönen R. Phenolic acids in berries, fruits, and beverages [J]. J Agric Food Chem, 2006, 54(19): 7193-7199.

[12] Clifford M N. Chlorogenic acids and other cinnamates – nature, occurrence and dietary burden [J]. Journal of the Science of Food & Agriculture, 1999, 79(3): 362-372.

[13] Pei K, Ou J, Huang J, et al. P-Coumaric acid and its conjugates: dietary sources, pharmacokinetic properties and biological activities [J]. Journal of the Science of Food & Agriculture, 2015, 96(9): 2952-2962.

[14] Martin-Tanguy J. The occurrence and possible function of hydroxycinnamoyl acid amides in plants [J]. Plant Growth Regulation, 1985, 3(3): 381-399.

[15] Shi, Y., Chen, Q. X., Wang, Q, et al.. Inhibitory effects of cinnamic acid and its derivatives on the diphenolase activity of mushroom (Agaricus bisporus) tyrosinase [J]. Food Chemistry, 2005, 92(4): 707–712.

[16] Spasova M, Philipov S, Nikolaeva-glomb L, et al. Cinnamoyl- and hydroxycinnamoyl amides of glaucine and their antioxidative and antiviral activities [J]. Bioorganic & Medicinal Chemistry, 2008, 16(15): 7457-7461.

[17] Lee S , Lee Choh J H , Kim E E , et al. Anti-atherogenic effects of 3,4-Dihydroxy hydrocinnamides [J]. Cheminform, 2003, 13(16): 2681-2682.

[18] Rajan P, Vedernikova I, Cos P, et al. Synthesis and evaluation of caffeic acid amides as antioxidants [J]. Bioorganic & Medicinal Chemistry Letters, 2001, 11(2): 215-217.

[19] Kim E O, Min K J, Kwon T K, et al. Anti-inflammatory activity of hydroxycinnamic acid derivatives isolated from corn bran in lipopolysaccharide-stimulated Raw 264.7 macrophages [J]. Food & Chemical Toxicology, 2012, 50(5): 1309-1316.

[20] Chuang S T, Kuo Y H, Su M J. KS370G, a caffeamide derivative, attenuates unilateral ureteral obstruction-induced renal fibrosis by the reduction of inflammation and oxidative stress in mice [J]. European Journal of Pharmacology, 2015, 750(1): 1-7.

[21] Kuo Y H, Wu P Y, Chen C W, et al. *N*-(4-bromophenethyl) caffeamide protects skin from UVB-induced inflammation through MAPK/IL-6/NF-ÎºB-dependent signaling in human skin fibroblasts and hairless mouse skin [J]. Molecules, 2017, 22(10): 1639.

[22] Hoshino S, Kurishima A, Inaba M, et al. Synthesis of amide compounds of ferulic acid, and their stimulatory effects on insulin secretion in vitro [J]. Bioorganic & Medicinal Chemistry, 2003, 11(17): 3807-3813.

[23] Wang D, Ma J F, Cai M X, et al. Synthesis and antioxidation of caffeic acid amino acid ethyl ester [J]. Fine Chemicals, 2019, 36(03): 494-498.

[24] Fan Q, Jiang H, Yuan E, et al. Tyrosinase inhibitory effects and antioxidative activities of novel cinnamoyl amides with amino acid ester moiety [J]. Food Chemistry, 2012, 134(2): 1081-1087.

[25] Hung C C, Tsai W J, Kuo L M, et al. Evaluation of caffeic acid amide analogues as anti-platelet aggregation and anti-oxidative agents [J]. Bioorg Med Chem, 2005, 13(5): 1791-1797.

[26] Treml J, Šmejkal K. Flavonoids as potent scavengers of hydroxyl radicals [J]. Comprehensive Reviews in Food Science & Food Safety, 2016, 15(4): 720-738.

[27] G mez-cordov S C, Bartolom B, Vieira W, et al. Effects of wine phenolics and sorghum tannins on tyrosinase activity and growth of melanoma cells [J]. Journal of Agricultural & Food Chemistry, 2001, 49(3): 1620-1624.

[28] Spasova M, Kortenska-kancheva V, Totseva I, et al. Synthesis of cinnamoyl and hydroxycinnamoyl amino acid conjugates and evaluation of their antioxidant activity [J]. Journal of Peptide Science, 2010, 12(5): 369-375.

[29] Wang D, Zhao X, Song J J, et al. Study on chemical modification of caffeic acid and antioxidation of products [J]. China Surfactant Detergent & Cosmetics, 2019, 49(02): 98-102.

[30] Anselmi C, Centini M, Andreassi M, et al. Conformational analysis: a tool for the elucidation of the antioxidant properties of ferulic acid derivatives in membrane models [J]. J Pharm Biomed Anal, 2004, 35(5): 1241-1249.

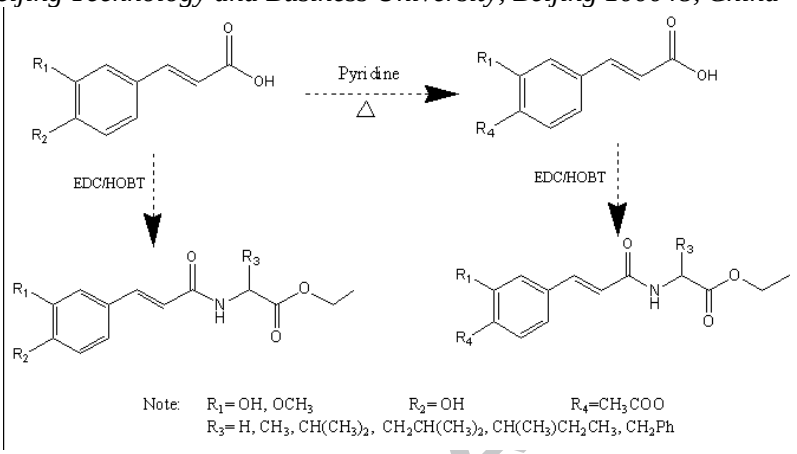
[31] Boath A S, Stewart D, M cdougall G J. Berry components inhibit α-glucosidase in vitro: synergies between acarbose and polyphenols from black currant and rowanberry [J]. Food Chemistry, 2012, 135(3): 929-936.

Graphical Abstract

Synthesis of N-hydroxycinnamoyl amide derivatives and evaluation of their anti-oxidative and anti-tyrosinase activities

Jun Zhu*, Dong Wang, etal

School of Science, Beijing Technology and Business University, Beijing 100048, China



Research Highlights

(i) Four new cinnamoyl amides with amino acid ester moiety were designed and synthesized.

(ii) The free radical-scavenging and anti-tyrosinase activities of the compounds were evaluated.

(iii) The antioxidant activity of compounds was due to the phenolic hydroxyl groups.

(iv) The tyrosinase inhibition activity of compouds mainly derive from the acetyl groups.