Free Radical Research, April 2011; 45(4): 445–453

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healthcare

Synthesis and antioxidant capacities of hydroxyl derivatives of cinnamoylphenethylamine in protecting DNA and scavenging radicals

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(Received date: 12 October 2010; Accepted date: 11 November 2010)

Abstract

Cinnamoylphenethylamine (CNPA) derivatives including feruloylphenethylamine (FRPA), caffeoylphenethylamine (CFPA), cinnamoyltyramine (CNTA), feruloyltyramine (FRTA) and caffeoyltyramine (CFTA) were synthesized in order to investigate the influence of the number and position of hydroxyl group on Cu^{2+}/glutathione (GSH) and 2,2'-azobis(2-amidinopropane hydrochloride) (AAPH)-induced oxidation of DNA. The radical-scavenging properties of these CNPA derivatives were also evaluated by trapping 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) cationic radical ($ABTS^{+\cdot}$), 2,2'-diphenyl-1-picrylhydrazyl radical (DPPH) and galvinoxyl radical. In addition, these CNPA derivatives were tested by linoleic acid (LH)-β-carotene-bleaching experiment. The chemical kinetic was employed to treat the results from AAPH-induced oxidation of DNA and gave the order of antioxidant ability as CFTA > CFPA > FRTA > FRPA. CFTA and CFPA also possessed high abilities to inhibit Cu^{2+}/GSH-mediated degradation of DNA, whereas FRPA and FRTA can protect LH against the autoxidation efficiently. Finally, CFPA and FRPA exhibited high activity in trapping $ABTS^{+\cdot}$, DPPH and galvinoxyl radicals. Therefore, the cinnamoyl group bearing *ortho*-dihydroxyl or hydroxyl with *ortho*-methoxyl benefited for CNPA derivatives to protect DNA, while hydroxyl in tyramine cannot enhance the radical-scavenging abilities of CNPA derivatives.

Keywords: *Cinnamoylphenethylamine, Ceratostigma willmottianum, free radical, oxidation, antioxidant, DNA*

Introduction

Ceratostigma willmottianum is widely used in Chinese folk medicine to treat rheumatism, traumatic injury and parotitis. The investigation on the chemical constituents in this plant reveals that cinnamoylphenethylamine (CNPA) is the major skeleton of the bioactive compounds [1]. The cinnamoyl group bridges with phenethylamine by amide linkage and the hydroxyl groups attach to benzene ring A and/or B to form a phenolic derivative of CNPA (see Scheme 1). Meanwhile, phenolic derivatives of CNPA such as feruloylphenethylamine (FRPA), caffeoylphenethylamine (CFPA), cinnamoyltyramine (CNTA), feruloyltyramine (FRTA) and caffeoyltyramine (CFTA) are also found in many other medicinal plants including *Peperomia duclouxii* [2], *Aristolochia constricta* [3] and *mollissima* [4], *Erycibe hainanesis* [5], *Physalis sordida* [6] and *Sparattanthelium tupiniquinorum* [7], etc. The pharmacological activities of these herbs including anti-tubercular [8], the anti-proliferation of HL-60, U937 and Jurkat cells [9] and the inhibition of the superoxide anion and elastase released from human neutrophils [10] were proved to correlate with phenolic derivatives of CNPA and their secondary metabolites [11]. More phenolic hydroxyl groups attaching to benzene ring A and B in CNPA enhance the ability to inhibit P-selectin and cyclooxygenase-I and -II expression [12], in which the mechanism of CFTA to inhibit cyclooxygenase-II expression was regarded to inhibit the activities of enhancer-binding protein and activator protein-1 transcription factors [13]. As a consequence, phenolic derivatives of CNPA even became popular ingredients of some commercial

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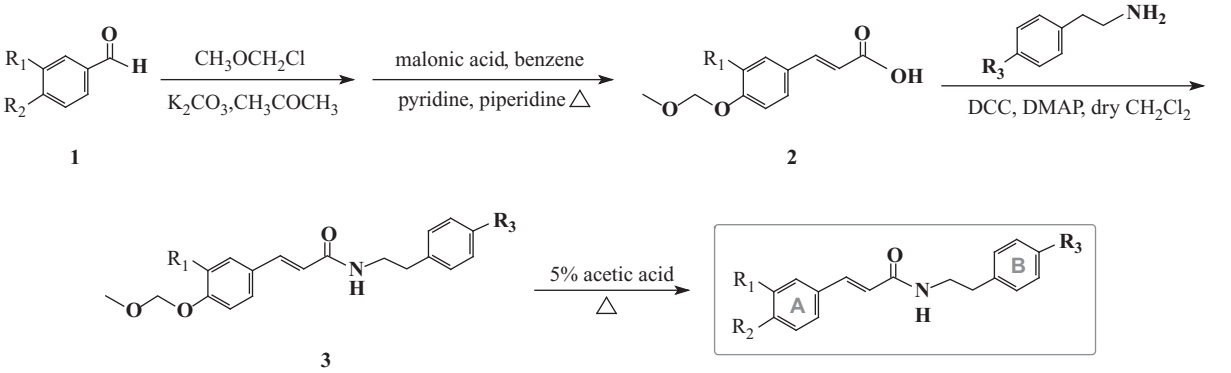
ISSN 1071-5762 print/ISSN 1029-2470 online
DOI: 10.3109/10715762.2010.540576



name	*abbreviation*	R_1	R_2	R_3
cinnamoylphenethylamine	**CNPA**	H	H	H
feruloylphenethylamine	**FRPA**	OCH_3	OH	H
caffeoylphenethylamine	**CFPA**	OH	OH	H
cinnamoyltyramine	**CNTA**	H	H	OH
feruloyltyramine	**FRTA**	OCH_3	OH	OH
caffeoyltyramine	**CFTA**	OH	OH	OH

CNPA and **CNTA**were prepared by cinnamic acid and corresponding amine in the presence of DCC, DMAP, and dry CH_2Cl_2.

Scheme 1. Synthesis routines of CNPA derivatives.

herb medicines [14]. In addition to phenolic hydroxyl groups trapping radicals and/or reducing oxidative species, N-H in the amide linkage in some drugs can also scavenge radicals directly [15]. Many works revealed the *in vivo* activities of CNPA derivatives, but the antioxidant behaviour of phenolic hydroxyl and amide groups in CNPA derivative were still necessary to be clarified *in vitro*. Especially, the investigation of the structure–activity relationship between hydroxyl group and radical-scavenging property was beneficial for understanding the antioxidant mechanism of CNPA derivatives. Therefore, as shown in Scheme 1, various hydroxyl-substituted CNPA derivatives were prepared in order to investigate the effects of hydroxyl and amide groups on the degradation of DNA, during which 2,2'-azobis (2-amidinopropane hydrochloride) (AAPH, R-N=N-R, R = $-CMe_2C(=NH)NH_2$) and Cu^{2+}/glutathione (GSH) acted as initiators of the oxidation of DNA, respectively. Moreover, the radical-scavenging properties of these compounds were also evaluated by interacting with 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate) cationic radical ($ABTS^{+}\cdot$), 2,2'-diphenyl-1-picrylhydrazyl (DPPH) and galvinoxyl radical, respectively. In addition, the abilities of these compounds to protect linoleic acid (LH) were screened by *β*-carotene-bleaching test.

Methods

Materials

2,2'-Azobis(2-amidinopropane hydrochloride), diammonium salt of 2,2'-azinobis(3-ethylbenzothiazoline-6-sulphonate), 2,2'-diphenyl-1-picrylhydrazyl, galvinoxyl radical, naked DNA sodium salt, glutathione, linoleic acid and *β*-carotene were purchased from ACROS ORGANICS (Geel, Belgium) and used as received. Other reagents were of analytical grade and purchased from Beijing Chemical Reagent Co. (Beijing, China). The structures of the obtained compounds were identified by NMR spectrometer (Varian Mercury 300). The spectra of the obtained compounds were listed in Supporting Information (Online version only). The obtained compounds were analyzed by HPLC, and the purities were larger than 98.0%.

Synthesis of cinnamoylphenethylamine derivatives

Synthesis of cinnamoylphenethylamine (CNPA) and cinnamoyltyramine (CNTA). CNPA was prepared by the amidation between 0.296 g (0.002 mol) of cinnamic acid and 0.28 mL of phenethylamine in 50 mL of dry CH_2Cl_2 with 0.452 g (0.0022 mol) of dicyclohexylcarbodiimide (DCC) as the dehydrant and 10 mg of *N*,*N*-dimethylaminopyridine (DMAP) as the catalyst. The aforementioned mixture was stirred at room temperature for 4 h to afford crude product, then the crude product was purified by silica chromatography (ethyl acetate:petroleum ether = 1:3) to give 0.424 g of CNPA, yield 84% and melting point: 121–122°C. ^{1}H NMR (300 MHz, DMSO-*d*6): δ (ppm) 2.80 (t, J = 7.2 Hz, 2H), 3.34–3.47 (m, 2H), 6.64 (dd, J = 3 Hz, J = 15.9 Hz, 2H), 7.18–7.33 (m, 5H), 7.36–7.47 (m, 4H), 7.55 (s, 1H), 7.69 (s, 1H), 8.20 (t, J = 4.8 Hz, 1H); ^{13}C NMR (75 MHz, DMSO-*d*6): δ (ppm) 35.1, 40.3, 122.2, 126.0, 127.4, 128.3, 128.6, 128.9, 129.3, 134.9, 138.5, 139.4, 164.9. CNTA was prepared by the amidation between 0.148 g (0.001 mol) of cinnamic acid and 0.206 g

(0.0015 mol) of tyramine in 150 mL of dry CH_2Cl_2 with 0.226 g (0.0011 mol) of DCC and 5 mg of DMAP added. The reaction mixture was stirred at room temperature for 4 h, and the crude product was purified by silica chromatography (ethyl acetate: chloroform = 1:4.5) to afford 0.165 g of CNTA, yield 62% and melting point: 188–189°C. ^{1}H NMR (300 MHz, DMSO-*d*6): δ (ppm) 2.65 (t, $\mathcal{J}$ = 7.35 Hz, 2H), 3.34 (t, $\mathcal{J}$ = 10.05 Hz, 2H), 6.59–6.69 (m, 3H), 7.00 (s, 1H), 7.03 (s, 1H), 7.34–7.43 (m, 4H), 7.54 (s, 1H), 7.56 (d, $\mathcal{J}$ = 1.5 Hz, 1H), 8.15 (t, $\mathcal{J}$ = 5.7 Hz, 1H), 9.18 (s, 1H); ^{13}C NMR (75 MHz, DMSO-*d*6): δ (ppm) 34.4, 40.7, 115.1, 122.3, 127.5, 128.9, 129.4, 129.5, 134.9, 138.5, 155.7, 164.9.

Synthesis of feruloylphenethylamine (FRPA) and caffeoylphenethylamine (CFPA). Vanillin (R_1 = OCH_3 and R_2 = OH in compound 1, 4.56 g, 0.03 mol) and K_2CO_3 (10.52 g, 0.076 mol) were mixed in acetone (70 mL) and stirred at room temperature for 15 min, to which 2.6 mL of CH_3OCH_2Cl was added dropwisely and stirred for at least 4 h. The reaction mixture was filtered to remove the solid phase and the solution was evaporated under vacuum to give intermediate product. Then, the obtained intermediate product was mixed with 5.86 g (0.056 mol) of malonic acid, 60 mL of benzene, 30 mL of pyridine and 0.9 mL of piperidine and refluxed for 8 h. After benzene was evaporated under vacuum, the residual mixture was acidified by HCl aqueous solution to pH = 1, a white solid (compound 2) was yielded (6.24 g, 87%). The compound 2 (2.38 g, 0.01 mol) was mixed with 1.4 mL of phenethylamine in 60 mL of dry CH_2Cl_2 with 2.26 g (0.011 mol) of DCC and 50 mg of DMAP added. The mixture was stirred at room temperature for 14 h and then cooled to precipitate DCC-H_2O complex. After the DCC-H_2O complex was removed by filtration and the solvent was evaporated under vacuum, the crude product was purified by silica chromatography (ethyl acetate:chloroform = 1:4.5) to afford 3.04 g of compound 3, yield 89%. The compound 3 was dissolved in 179 mL of 5% acetic acid aqueous solution and refluxed for 12 h. After the mixture was cooled to room temperature, the pH value of the mixture was adjusted to 7 by adding saturated $NaHCO_3$ aqueous solution, then extracted by ethyl acetate three times. The ethyl acetate phase was washed with saturated NaCl solution and dried over Na_2SO_4. Ethyl acetate was evaporated under vacuum and the crude product was purified by silica chromatography (ethyl acetate:chloroform = 1:3) to afford 2.1 g of FRPA, yield 79% and melting point: 33.5–35°C. ^{1}H NMR (300 MHz, DMSO-*d*6): δ (ppm) 2.69–2.79 (m, 2H), 3.36–3.41 (m, 2H), 3.74–3.84 (m, 3H), 6.43 (d, $\mathcal{J}$ = 15.9 Hz, 1H), 6.78 (dd, $\mathcal{J}$ = 2.7 Hz, $\mathcal{J}$ = 8.1 Hz, 1H), 6.98 (dd, $\mathcal{J}$ = 3 Hz, $\mathcal{J}$ = 8.1 Hz, 1H), 7.10 (s, 1H), 7.16–7.34 (m, 6H), 8.01 (t, $\mathcal{J}$ = 5.7 Hz, 1H), 9.40 (m, 1H); ^{13}C NMR (75 MHz, DMSO-*d*6): δ (ppm) 35.2, 40.3, 55.5, 110.8, 115.6, 119.0, 121.5, 126.0, 126.4, 128.3, 128.6, 138.9, 139.5, 147.8, 148.3, 165.4. CFPA was derived from FRPA by demethylation. Briefly, 0.596 g (0.002 mol) of FRPA was dissolved in 10 mL of dry CH_2Cl_2, then 0.374 g of $AlCl_3$ and 10 mg of triethylbenzyl ammonium chloride (TEBA) were added and stirred at 5°C in an ice-bath. Finally, 0.53 mL of (0.0066 mol) pyridine was added dropwisely under stirring. The mixture was refluxed for 28 h and cooled to room temperature. The pH value of the mixture was adjusted to 1 by adding HCl aqueous solution to produce yellow solid. The mixture was extracted by ethyl acetate. Ethyl acetate phase was washed by saturated NaCl solution and dried over Na_2SO_4. After ethyl acetate was evaporated under vacuum, the crude product was purified by silica chromatography (ethyl acetate:chloroform = 1:2) to afford 0.47 g of CFPA, yield 81% and melting point: 148–150°C. ^{1}H NMR (300 MHz, DMSO-*d*6): δ (ppm) 2.77 (t, $\mathcal{J}$ = 14.7 Hz, 2H), 3.39 (q, $\mathcal{J}$ = 13.2 Hz, 2H), 6.33 (dd, $\mathcal{J}$ = 3.6 Hz, $\mathcal{J}$ = 15.3 Hz, 1H), 6.74 (d, $\mathcal{J}$ = 8.1 Hz, 1H), 6.83 (m, 1H), 6.94 (d, $\mathcal{J}$ = 1.5 Hz, 1H), 7.17–7.32 (m, 6H), 8.05 (t, $\mathcal{J}$ = 5.6 Hz, 1H), 9.10 (s, 1H), 9.33 (s, 1H); ^{13}C NMR (75 MHz, DMSO-*d*6): δ (ppm) 35.2, 40.3, 113.8, 115. 7, 118.5, 120.3, 126.0, 126.4, 128.3, 128.6, 139.0, 139.5, 145.5, 147.2, 165.3.

Synthesis of feruloyltyramine (FRTA) and caffeoyltyramine (CFTA). Tyramine was used in the synthesis of FRTA and CFTA and the other operation was similar to the synthesis of FRPA and CFPA. Briefly, 0.238 g (0.001 mol) of compound 2, 0.226 g (0.0011 mol) of DCC and 5 mg of DMAP were dissolved in 10 mL dry CH_2Cl_2 and added to tyramine solution (0.206 g of tyramine dissolved in 100 mL of dry CH_2Cl_2) within 4 h. The mixture was stirred for another 4 h and then cooled to precipitate DCC-H_2O complex. After the DCC-H_2O complex was removed by filtration and the solution was washed by NaOH solution (1 M), the pH value of the water phase was adjusted to 1 by adding HCl aqueous solution to yield white solid. The mixture was extracted by ethyl acetate. Ethyl acetate phase was washed by saturated NaCl solution and dried over Na_2SO_4. After ethyl acetate was evaporated under vacuum, the crude product was dissolved in 50 mL of 5% acetic acid and refluxed for 4 h. The following treatment was similar to the synthesis of FRPA, affording 0.204 g of FRTA, yield 57% and melting point: 59–61°C. ^{1}H NMR (300 MHz, DMSO-*d*6): δ (ppm) 2.65 (t, $\mathcal{J}$ = 7.2 Hz, 2H), 3.34 (s, 2H), 3.80 (s, 3H), 6.43 (d, $\mathcal{J}$ = 15.6 Hz, 1H), 6.67 (s, 1H), 6.69 (s, 1H), 6.79 (d, $\mathcal{J}$ = 8.1 Hz, 1H), 6.97–7.03 (m, 3H), 7.11 (s, 1H), 7.31 (d, $\mathcal{J}$ = 15.6 Hz, 1H), 7.96 (t, $\mathcal{J}$ = 4.8 Hz, 1H), 9.15 (s, 1H), 9.39 (s, 1H); ^{13}C NMR (75 MHz,

DMSO-*d*6): δ (ppm) 34.4, 40.6, 55.5, 110.8, 115.1, 115.6, 119.1, 121.5, 126.4, 129.4, 129.5, 138.8, 147.8, 148.2, 155.6, 165.3. CFTA was derived from FRTA by demethylation. Briefly, 0.314 g of FRTA, 0.187 g of $AlCl_3$ and 5 mg of TEBA gave 0.254 g of CFTA, yield 85% and melting point: 199.5–201°C. ^{1}H NMR (300 MHz, DMSO-*d*6): δ (ppm) 2.64 (t, $\mathcal{J}$ = 7.5 Hz, 2H), 3.28–3.30 (m, 2H), 6.31 (d, $\mathcal{J}$ = 15.6 Hz, 1H), 6.66–6.75 (m, 3H), 6.83 (dd, $\mathcal{J}$ = 21 Hz, $\mathcal{J}$ = 8.4 Hz, 1H), 6.93 (d, $\mathcal{J}$ = 2.1 Hz, 1H), 7.00 (d, $\mathcal{J}$ = 2.1 Hz, 1H), 7.02 (d, $\mathcal{J}$ = 1.8 Hz, 1H), 7.22 (d, $\mathcal{J}$ = 15.9 Hz, 1H), 8.02 (t, $\mathcal{J}$ = 5.7 Hz, 1H), 9.13 (s, 1H), 9.18 (s, 1H), 9.36 (s, 1H); ^{13}C NMR (75 MHz, DMSO-*d*6): δ (ppm) 34.5, 40.7, 113.8, 115.1, 115.7, 118.6, 120.4, 126.4, 129.5, 129.5, 138.9, 145.5, 147.2, 155.6, 165.3.

Cu^{2+}/GSH or AAPH-induced oxidation of DNA

The oxidation of DNA mediated by Cu^{2+} and GSH was following our previous description [16]. The phosphate buffered solution (PBS_1: 6.1 mM Na_2HPO_4 and 3.9 mM NaH_2PO_4) was applied to dissolve $CuSO_4$, DNA and GSH. The final concentrations of DNA, Cu^{2+} and GSH were 2.0 mg/mL, 5.0 mM and 3.0 mM, respectively, to which dimethyl sulphoxide (DMSO) solutions of CNPA derivatives were added to a final concentration of 200 μM, respectively. The total volume of the solution was 15 mL and the volume of DMSO was 0.1 mL. This solution was dispensed into test tubes, each one contained 2.0 mL. The test tubes were incubated in a water bath (37°C) to initiate the oxidation of DNA. Three tubes were taken out at every 30 min and cooled immediately. To each tube, 1.0 mL of 30.0 mM PBS_1 solution of EDTA, 1.0 mL of thiobarbituric acid (TBA) solution (1.00 g TBA and 0.40 g NaOH dissolved in 100 mL of PBS_1, the addition of NaOH was beneficial for TBA dissolving in aqueous solution) and 1.0 mL of 3.0% trichloroacetic acid aqueous solution were added and heated in boiling water for 30 min. After the solution was cooled to room temperature, 1.5 mL of *n*-butanol was added and shaken vigorously to extract TBA reactive substance (TBARS). The absorbance of the *n*-butanol layer was measured at 535 nm and is shown in Supporting Information (Online version only).

AAPH-induced oxidation of DNA was following our previous description [16]. The phosphate buffered solution (PBS_2: 8.1 mM Na_2HPO_4, 1.9 mM NaH_2PO_4, 10.0 μM EDTA) was applied to dissolve DNA and AAPH. The final concentrations of DNA and AAPH were 2.0 mg/mL and 40.0 mM, respectively. CNPA derivatives were dissolved in DMSO and added to the aforementioned solution to reach various concentrations. The total volume of the solution was 15 mL and the volume of DMSO was 0.1 mL. This solution was dispensed into test tubes, each one contained 2.0 mL. The test tubes were incubated in a water bath (37°C) to initiate the oxidation of DNA. Three tubes were taken out at every 2 h and cooled immediately. The following operation was the same as the oxidation of DNA mediated by Cu^{2+}/GSH except that the heating period was 15 min after TBA and trichloroacetic acid aqueous solution were added.

β-Carotene-bleaching test

An emulsion was prepared by dissolving 5.0 mg of β-carotene, 40 mg of LH and 400 mg of Triton X-100 in 5.0 mL of $CHCl_3$. After $CHCl_3$ was evaporated under vacuum pressure, 100 mL of oxygen-saturated water was added and the mixture was shaken under ultrasonic vibration to form a homogeneous β-carotene-LH emulsion (λ_{max} = 460 nm) [17]. The ethanol solutions of CNPA derivatives (0.1 mL) were mixed with 1.9 mL of β-carotene-LH emulsion, in which the final concentrations of CNPA derivatives were 20.0 μM. The decay of the absorbance at 460 nm was measured.

Scavenging radicals

The experiments of CNPA derivatives to trap $ABTS^{+\cdot}$, DPPH and galvinoxyl radical were following the description in the literature [18,19]. Briefly, ABTS (2.00 mL, 4.0 mM) was oxidized by 1.41 mM $K_2S_2O_8$ for 16 h to generate $ABTS^{+\bullet}$, to which 100 mL of ethanol was added to make the absorbance (Abs_{ref}) around 0.70 at 734 nm ($\varepsilon_{734} = 1.6 \times 10^4$ $M^{-1}cm^{-1}$, the concentration of $K_2S_2O_8$ was lower than that of ABTS in order to verify complete exhaustion of $K_2S_2O_8$). DPPH and galvinoxyl radical were dissolved in ethanol to make the absorbance (Abs_{ref}) around 1.00 at 517 nm ($\varepsilon_{517} = 4.09 \times 10^3$ $M^{-1}cm^{-1}$) and 428 nm ($\varepsilon_{428} = 1.4 \times 10^5$ $M^{-1}cm^{-1}$), respectively. The ethanol solution of CNPA derivatives were added to the aforementioned radical solutions to the final concentration of 20.0 μM. The absorbance of the mixture (Abs_{detect}) was recorded at room temperature and the concentrations of the residual radical species were calculated by the products of Abs_{detect} and the corresponding ε. The concentrations of radical species were plotted vs incubation time.

Statistical analysis

All the data were the average values from at least three independent measurements with the experimental error within 10%. The data were analysed by one-way ANOVA on Origin 6.0 professional Software and $p < 0.001$ indicated a significance difference.

Results

Inhibitive effects of CNPA derivatives on Cu^{2+}/GSH-induced degradation of DNA

Cu(II) is able to oxidize GSH to form Cu(I) and GS· and then Cu(I) combines with DNA to form DNA-Cu(I) complex. GS· may interact with the complex, leading to the degradation of DNA to form carbonyl species [16]. The carbonyl species can be detected after reacting with thiobarbituric acid (TBA). DNA (2.0 mg/mL), Cu^{2+} (5.0 mM) and GSH (3.0 mM) were mixed in PBS_1 and incubated at 37°C for 3 h. The absorbance of TBA reactive substance (TBARS, λ_{max} = 535 nm) was assigned as 100% and the absorbances of TBARS in the presence of 200 μM CNPA derivatives were compared with that in the blank experiment and outlined in Figure 1.

The percentages of TBARS in the presence of CNPA derivative were CFTA < CFPA < CNTA < FRTA < FRPA < CNPA < Blank. The percentages of CNPA (98.4%), FRPA (97.7%) and FRTA (96.0%) were higher than those of other CNPA derivatives, revealing that the abilities of these CNPA derivatives to protect DNA against Cu^{2+}/GSH-induced degradation were lower than those of CNTA, CFPA and CFTA. Moreover, the lowest ability of CNPA implied that N-H in amide was not active to Cu^{2+}/GSH-induced degradation of DNA. The ability of FRPA was similar to that of CNPA, indicating that the hydroxyl group in combination with *ortho*-methoxyl cannot improve the protective capacity of CNPA derivatives in this case. CFPA and CFTA possessed remarkably higher abilities than other CNPA derivatives, demonstrating that two hydroxyl groups at *ortho*-position along with tyramine enhanced the inhibitive effect significantly. This may be due to the two hydroxyl groups being able to chelate Cu^{2+}, resulting in less GS· and DNA-Cu(I) complex being produced. Consequently, DNA cannot be destroyed as much as in the blank experiment.

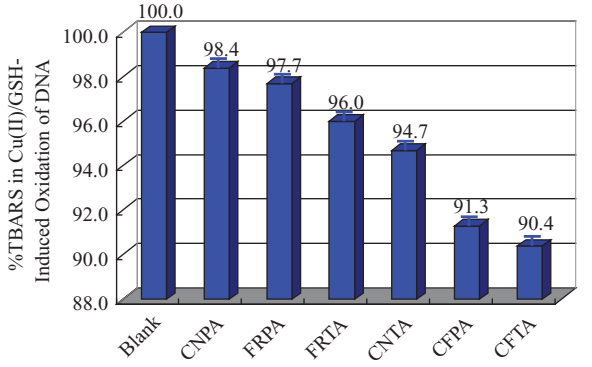


Figure 1. Percentages of TBARS in the mixture of DNA (2.0 mg/mL), Cu^{2+} (5.0 mM), GSH (3.0 mM) and CNPA derivatives (200 μM) when the mixture was incubated at 37°C for 3 h.

Antioxidant capacities of CNPA derivatives in AAPH-induced oxidation of DNA

AAPH is a resource of peroxyl radical (ROO·, $R=-CMe_2C(=NH)NH_2$) for abstracting H atom from the C-4' atom of DNA, resulting in the cleavage of DNA and the formation of carbonyl species eventually [16]. It can be found in Figure 2 that the absorbance of TBARS increased with the incubation period in the blank experiment, indicating that more carbonyl species were generated from the degradation of DNA with the increase of the incubation period.

The additions of various concentrations of CNPA cannot influence the increase of the absorbance of TBARS, demonstrating that N-H in CNPA was not able to hinder AAPH-induced oxidation of DNA. The absorbance of TBARS decreased remarkably when other CNPA derivatives were added to the above experimental system, revealing that the hydroxyl group attaching to CNPA enhanced its antioxidant ability to protect DNA against AAPH-induced oxidation. In particular, as shown in Figure 2, when FRPA, FRTA, CFPA and CFTA were added, the increase of the absorbance of TBARS was retarded for a period at the beginning of the incubation and then increased as that in the blank experiment. This period was designated as the inhibition period (t_{inh}) and was prolonged with the increase of the concentrations of these four compounds. Figure 3 outlined the linear relationship between the concentrations of these four compounds and t_{inh} and the equations of t_{inh} ~ [FRPA, FRTA, CFPA and CFTA] were listed in Table I.

In the equations in Table I, the coefficient indicated the sensitivity of t_{inh} to the variety of the concentration of CNPA derivatives and the constants were generated in the linear regression to balance the equation. The high coefficient meant that a little change of the concentration of CNPA derivatives led to remarkable variety of the t_{inh}. Thus, the order of the coefficient, CFTA > CFPA > FRTA > FRPA, indicated that the increase of the concentration of CFTA resulted in the increase of t_{inh} more significantly than other compounds. This may be that high concentration of CFTA can scavenge the peroxyl radicals of AAPH and thereby cause inhibition, while CNTA is not a peroxyl radical scavenger. Thus, CFTA was a concentration-dependent antioxidant with high efficiency in this case. The addition of CNTA actually decreased the absorbance of TBARS, but CNTA cannot generate t_{inh}, even high concentration was applied. Comparing the structure of CNTA with that of CFTA it can be concluded that more hydroxyl groups enhanced the antioxidant effectiveness of CNPA derivatives remarkably.

Effects of CNPA derivatives on β-carotene-bleaching test

LH and β-carotene can form a water-soluble emulsion in the presence of Triton X-100 or Tween as the

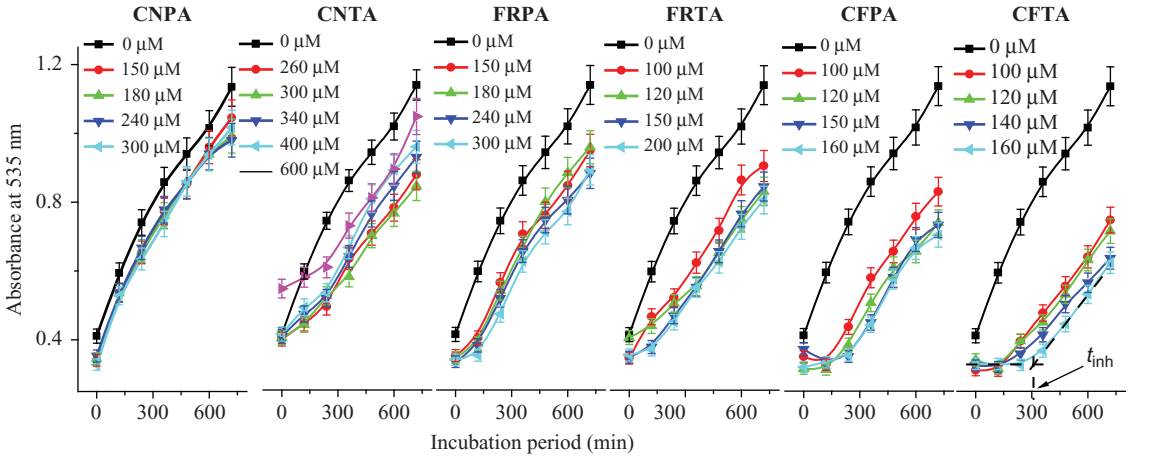


Figure 2. The absorbance of TBARS in the mixture of DNA (2.0 mg/mL), AAPH (40 mM) and various concentrations of CNPA derivatives at 37°C.

surfactant [17]. The auto-oxidation of LH led to the formation of peroxyl radical of LH (LOO·) that can blench *β*-carotene. As can be seen in Figure 4, the absorbance of *β*-carotene at 460 nm decreased continuously, indicating that much more LH was oxidized by the ambient oxygen with the incubation period increasing.

Other lines in Figure 4 illustrated the decrease of the absorbance at 460 nm in the presence of 20 μM CNPA derivatives. It can be found that the lines of CNPA and CNTA were even below the line of the blank experiment, implying that the addition of CNPA and CNTA accelerated the auto-oxidation of LH and CNPA and CNTA played a pro-oxidative role in this case. The lines of CFPA and CFTA (superposition) located above the blank line, indicating that CFPA and CFTA showed similar activity in inhibiting the auto-oxidation of LH. Furthermore, the additions of FRTA and FRPA efficiently hindered the decay of the absorbance, revealing that FRTA, especially FRPA, protected LH against the auto-oxidation significantly. This may be ascribed to hydroxyl with an *ortho*-methoxyl group. The present data also implied that *ortho*-dihydroxyl groups and the hydroxyl group in tyramine were not powerful enough groups to inhibit the auto-oxidation of LH.

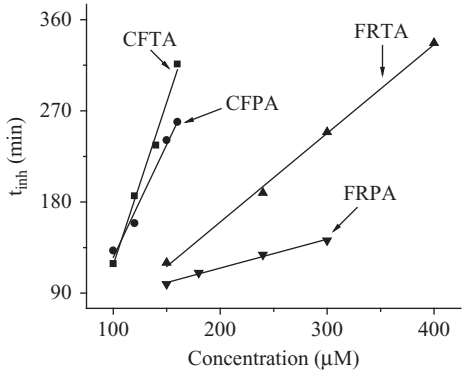


Figure 3. The relationship between the concentrations of CFTA, CFPA, FRTA and FRPA and inhibition period (t_{inh}).

Radical-scavenging abilities of CNPA derivatives

$ABTS^{+\bullet}$, DPPH and galvinoxyl are stable radicals at room temperature. The ability of the phenolic hydroxyl group in an antioxidant to reduce radicals can be measured by the interaction of the antioxidant with $ABTS^{+\bullet}$. The interactions of an antioxidant with DPPH and galvinoxyl radicals show the ability of the antioxidant to donate its hydrogen atom to N- and O-centred radicals, respectively. Figure 5 outlined the decay of the radical concentrations when ethanol solutions of CNPA derivatives (20.0 μM) were mixed with $ABTS^{+\bullet}$, DPPH and galvinoxyl solutions, respectively.

Recently, a novel method has been reported to treat the data of antioxidants to trap $ABTS^{+\bullet}$ [18]. On the basis of chemical kinetic, the decay of $[ABTS^{+\bullet}]$ along with the incubation period (t) can be expressed by equation (1).

$$[ABTS^{+\bullet}] = [ABTS^{+\bullet}]_{\infty} + \frac{ab}{b+t} \quad (1)$$

where $a = n_{app}[AH]_0$ and $b = 1/(k_2{}^{app}[AH]_0)$. $[ABTS^{+\bullet}]_{\infty}$ is the residual concentration of $ABTS^{+\bullet}$ when the

Table I. The relationships between the concentrations of CFTA, CFPA, FRTA and FRPA and t_{inh} and stoichiometric factor (n) of the aforementioned compounds.[a]

Antioxidant	t_{inh} (min) = (n/R_i) [CNPA derivatives (μM)] + constant	n
CFTA	t_{inh} = 3.20 [CFTA] – 202.4	10.8
CFPA	t_{inh} = 2.12 [CFPA] – 85.1	7.1
FRTA	t_{inh} = 0.88 [FRTA] – 10.4	3.0
FRPA	t_{inh} = 0.29 [FRPA] + 56.6	1.0

[a]$R_i = R_g = 1.4 \times 10^{-6} \times 40$ mM/s = 3.36 μM/min when 40 mM AAPH was employed to initiate the oxidation of DNA.

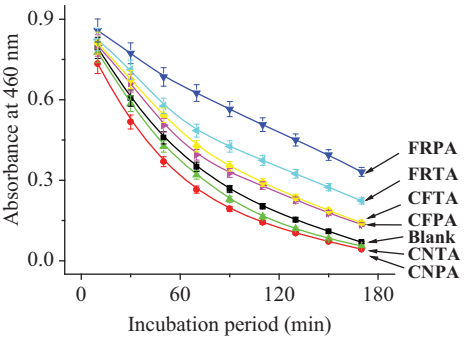


Figure 4. Decay of the absorbance at 460 nm of β-carotene-LH emulsion containing 20.0 μM CNPA derivatives.

reaction time (t) approaches to ∞ and $[AH]_0$ refers to the initial concentration of the antioxidant. Thus, n_{app}, the apparent stoichiometric factor, means the number of electrons that the antioxidant traps radicals, while k_2^{app} is the apparent rate constant of the limiting step of the reaction between the antioxidant and $ABTS^{+\bullet}$. Traditionally, effective concentration (EC_{50}) refers to the concentration of the antioxidant applied to scavenge 50% concentration of the radical, while T_{EC50} means the time needed to reach the steady state at EC_{50}. According to the kinetic deduction, $1/(k_2^{app}[AH]_0)$ represents the time necessary for the radicals to be decreased to 50% and, thus, has the same physical meaning as T_{EC50} [18]. Therefore, n_{app} and T_{EC50} can be obtained when t and the corresponding $[ABTS^{+\bullet}]$ are input into software to obtain equation (1). The obtained n_{app} is a thermodynamic parameter to express the ability of the antioxidant in trapping radicals and T_{EC50} is a kinetic parameter to indicate the reaction rate between the antioxidant and radicals. Because the same initial concentration of CNPA derivatives were employed herein, n_{app} and T_{EC50} as thermodynamic and kinetic parameters can be used to characterize the radical-scavenging activities of CNPA derivatives. Furthermore, the results from the interactions between CNPA derivatives and DPPH or galvinoxyl radical were also treated by the aforementioned method and n_{app} and T_{EC50} in scavenging three kinds of radical were listed in Table II.

The high value of n_{app} indicates that the antioxidant is able to provide many electrons to trap radicals and low value of T_{EC50} implicates that the antioxidant can trap radicals much more rapidly. Taking n_{app} and T_{EC50} into consideration, one can find that FRPA and CFPA were able to trap $ABTS^{+\bullet}$ and DPPH efficiently, while CFPA and CFTA showed high effectiveness to scavenge galvinoxyl radicals. In particular, the lowest value of n_{app} of CNPA indicated that N-H in CNPA possessed very weak ability to trap radicals. The relative low value of n_{app} of CNTA revealed that hydroxyl group in tyramine was not active to trap radicals, whereas hydroxyl with an *ortho*-methoxyl group and *ortho*-dihydroxyl groups exhibited powerful effectiveness to trap radicals.

Discussion

CNPA derivatives attracted much research attention because they were found in many folk medicinal herbs. As a kind of simple molecule, hydroxyl-substituted CNPA derivatives were also applied to explore the interactions between drug and receptor [20] or proteins [21]. In addition to the study on the biosynthetic pathway of CNPA derivatives [22], some artificial synthetic routines were designed on the basis of the amidation of phenethylamine by using cinnamic acid [23] or cinnamoyl anhydride [24]. In the direct amidation of phenethylamine and cinnamic acid, some tertiary amines were added to ionize the acid. For example, benzotriazol-1-yloxy-tri(dimethylamino)phosphonium hexafluorophosphate was used to catalyse the amidation of *p*-hydroxycinnamic acid derivatives with phenylalkylamines [25]. *N*,*N*-Dimethylaminopyridine (DMAP), a simple tertiary amine, was employed herein because of its high activity to couple phenethylamine or tyramine with cinnamic acid, as well as dicyclohexylcarbodiimide (DCC) was used to improve the dehydration between -COOH and $-NH_2$ according to the literature [23]. In our work, the hydroxyl in ferulic acid was etherized by CH_3OCH_2Cl before the amidation because it was very sensitive to the oxidation and the synthesis was carried out at room temperature to obtain satisfied yield.

The N-H bond in CNPA exhibited very low activity to scavenge radicals (data shown in Table II) and the data of CNTA showed that the radical-scavenging ability was not remarkably improved when the benzene ring B (see Scheme 1) was replaced by tyramine. The antioxidant behaviour of tyramine, as pointed out in the literature [26], was owing to the deamination to form *p*-hydroxyphenylacetaldehyde and/or to couple with another tyramine at *ortho*-position of hydroxyl group to form dimmer of tyramine. On the other hand, as shown in Table II, the values of n_{app} of FRPA and CFPA were higher than those of FRTA and CFTA. This result was in agreement with the traditional conclusion that hydroxyl with an *ortho*-methoxyl group and *ortho*-dihydroxyl group played the major role in scavenging radicals. On the other hand, our results indicated that the introduction of tyramine decreased the abilities of CNPA derivatives to trap radicals. However, as pointed out in the literature [27], the abilities of CNPA derivatives to inhibit U937 and Jurkat cells may be enhanced by replacing tyramine by dopamine to form caffeoyldopamine because *ortho*-dihydroxyl group attached to two benzene rings. Moreover, CNPA derivatives with *ortho*-dihydroxyl group attached to benzene ring A benefitted for chelating metal ions and, consequently, showed relative high activities to inhibit metal ion-mediated oxidation of DNA (see Figure 1).

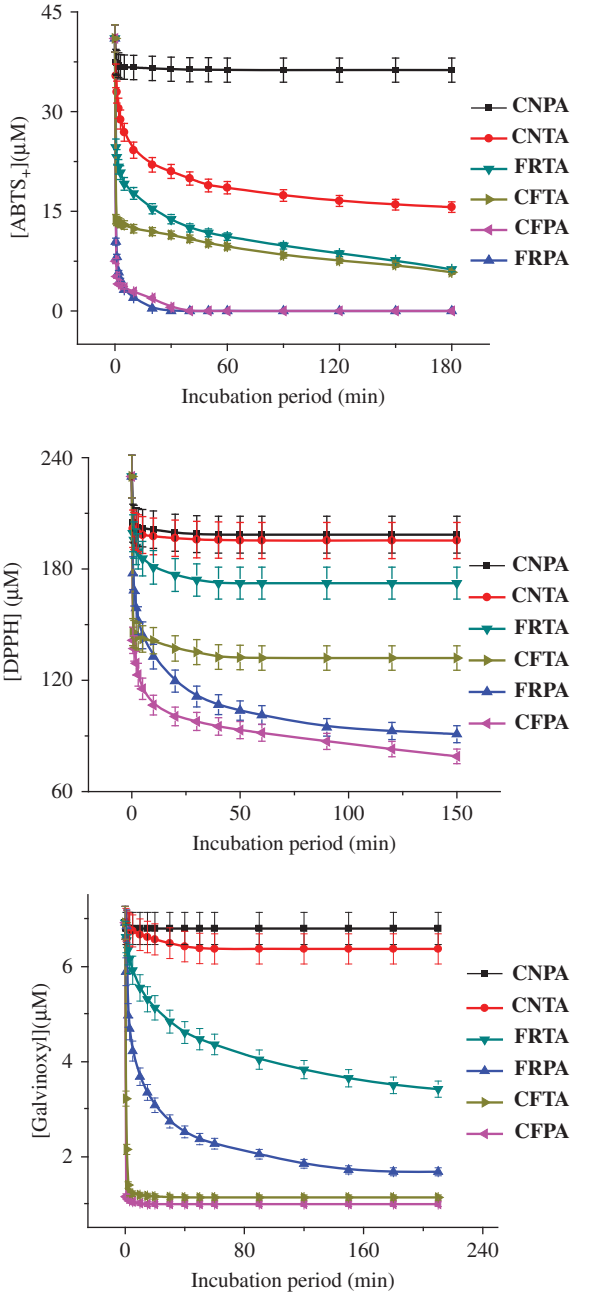


Figure 5. Decay of the absorbance of $ABTS^{+\cdot}$, DPPH and galvinoxyl radical solution containing 20.0 μM CNPA derivatives.

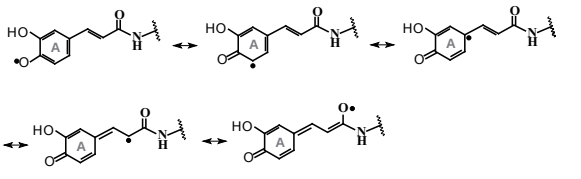
The chemical kinetic deduction reveals the relationship between the inhibition period (t_{inh}) and the concentration of the antioxidant (AH) as equation (2) [28].

$$t_{inh} = (n/R_i)\ [AH] \qquad (2)$$

where n is *stoichiometric factor* to express the ability of the antioxidant to terminate the radical-chain propagation and R_i refers to the initiation rate of the radical-induced reaction. It has been proved that this equation was also available for evaluating the antioxidant capacities in biological experimental system [29]. We have assumed that the rate of initiating the radical propagation was equal to that of the radical generated from the decomposition of AAPH ($R_g = 1.4 \times 10^{-6}$ [AAPH] s^{-1} [29]), *viz.*, $R_i = R_g = 1.4 \times 10^{-6} \times 40$ mM/s = 3.36 μM/min when 40 mM AAPH was employed to initiate the oxidation of DNA [30]. Thus, n values of CNPA derivatives can be calculated because the coefficients in the equations of t_{inh} ~ [CNPA derivatives] were n/R_i, and the results were involved in Table I. Higher values of n of CFTA and CFPA revealed that the *ortho*-dihydroxyl group benefitted for CNPA derivatives to protect DNA against AAPH-induced oxidation. The n value of CFTA was higher than that of CFPA and the same order of the n values was also found in FRTA and FRPA, indicating that replacing the benzene ring by tyramine benefitted for the enhancement of the antioxidant effectiveness in this case. All the CNPA derivatives possess radical-scavenging properties, thus, the protective effects of these compounds may be due to the scavenge peroxyl radical deriving from the decomposition of AAPH in protecting DNA against AAPH-induced oxidation. Since two hydroxyl groups attach to ring A in CFTA and CFPA, and ring A forms a conjugation system with the C=C bond in the carbon chain, radicals deriving from the *ortho*-dihydroxyl group in CFTA and CFPA may be stabilized by the conjugation system *via* resonance structure, as shown in equation (3). But the radical deriving from the hydroxyl group at ring B cannot be stabilized by the conjugation system. Thus, hydroxyl groups at ring A play main role in trapping radicals.

Table II. The apparent stoichiometric factor (n_{app}) and T_{EC50} of CNPA derivatives in trapping $ABTS^{+\cdot}$, DPPH and galvinoxyl radicals.

	Reacting with $ABTS^{+\cdot}$		Reacting with DPPH		Reacting with galvinoxyl	
Antioxidant	n_{app}	T_{EC50} (min)	n_{app}	T_{EC50} (min)	n_{app}	T_{EC50} (min)
CNPA	0.23	0.22	1.52	0.15	0.01	—
CNTA	1.10	3.95	1.68	0.13	0.03	13.36
FRPA	2.03	0.21	5.78	2.11	0.25	5.88
FRTA	1.37	1.16	2.69	0.78	0.17	25.60
CFPA	2.01	0.12	6.61	0.45	0.30	0.02
CFTA	1.57	0.12	4.71	0.09	0.29	0.24



(3)

Conclusion

More hydroxyl groups enhanced the antioxidant capacities of CNPA derivatives. Especially, *ortho*-dihydroxyl or hydroxyl with *ortho*-methoxyl attaching to the

benzene ring in cinnamoyl group increased the protective abilities of CNPA derivatives to protect DNA against radical and metal ion-mediated oxidation. The hydroxyl in tyramine, however, cannot significantly increase the radical-scavenging abilities of CNPA derivatives. The information obtained in this work may be useful for the design of CNPA-related antioxidants.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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This paper was first published online on Early Online on 9 December 2010.

Supplementary material available online

Figure S1.