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Synthesis and evaluation of novel ascorbyl cinnamates as potential anti-oxidant and antimicrobial agents

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Abstract An efficient preparation procedure has been proposed for the synthesis of 5,6-di-O-cinnamyl-L-ascorbic acid ester (5,6-CA–AA) and 2-O-cinnamyl-L-ascorbic acid ester (2-CA–AA) via ascorbic acid (AA) and cinnamic acid (CA), and their anti-oxidant and antibacterial activities were also investigated. Among the factors affecting the synthesis efficiency of 5,6-CA–AA and 2-CA–AA, reaction solvent and substrate molar ratio were very important, resulting in 76 and 54% total yield, respectively. Further studies indicated that 5,6-CA–AA maintained the inherent inhibitory ability of AA in α, α-diphenyl-β-picrylhydrazyl radical scavenging assay. Also, it had stronger scavenging activity against hydroxyl radicals than that of standard tert-butylhydroquinone (EC_{50}: 7.84 vs. 8.38 mM). In addition, 5,6-CA–AA and 2-CA–AA possessed comparable antibacterial activity to that of CA. Significantly, the application of ascorbyl cinnamate could retard the lipid oxidation of peanut oil and pork, and the corresponding perioxide value and thiobarbituric acid reactive substance content decreased by 32.0 and 56.5%, respectively. Moreover, the microbial reproduction of the pork treated with mixed ascorbyl cinnamate was attenuated to some extent. These investigations will pave the way for designing more efficient bifunctional derivatives of AA and CA.

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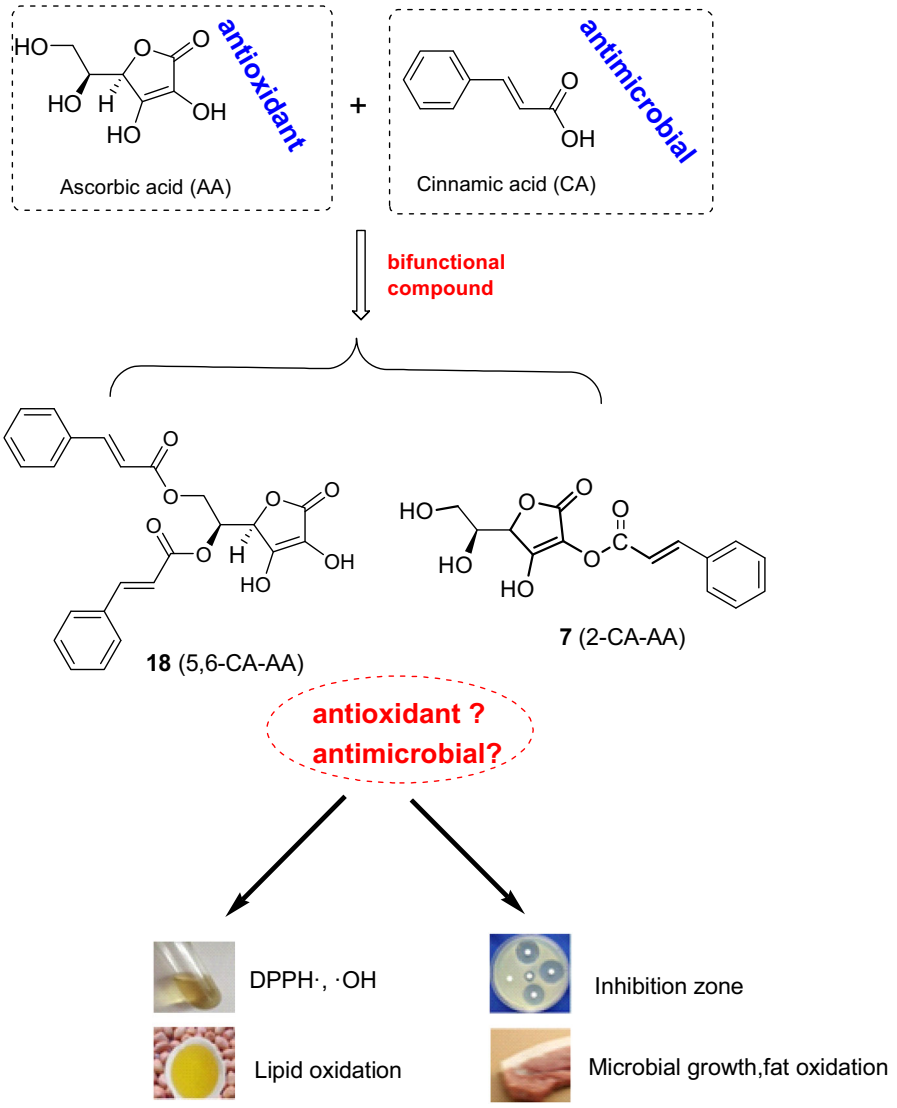
Graphical Abstract



Keywords Ascorbic acid · Cinnamic acid · Anti-oxidant activity · Antibacterial activity · Lipid oxidation

Introduction

L-ascorbic acid (AA) is an important anti-oxidant in drug, food and biological systems [1], but it is easily soluble and unstable under aqueous conditions, which hinder its application in lipid condition. To overcome this problem, AA has been chemically modified by protecting alcohol functions or the enediol system. Among these derivatives, 2-O-α-D-glucopyranosylascorbic acid **1** and salts of ascorbyl 2-phosphoric esters **2** (Fig. 1) have been successfully used in cosmetics and food additives [2, 3]. Moreover, some active substances were linked at the C-2 or C-3 hydroxyl group of AA so as to demonstrate multiple biological properties. AA/kojic acid hybrids **3** [4] and AA/ferulic ester hybrids **4–6** [5] were part of them. Also, Galey developed the preparation of 2-O-ascorbyl cinnamate derivatives **7–10** (Fig. 2) and diesters of AA such as 2-O-[6-palmitoyl ascorbyl]-4′-acetoxy ferulate **11**, and their protective properties against oxidation of lipid constituents of the skin have also been claimed [6].

Compared with those modified in the enediol position, the derivatives modified in the alcohol position of AA have been widely investigated, especially the potential bifunctional compound. ascorbyl benzoate **12** (Fig. 3) was achieved through lipase-catalysis in cylcohexanone, and its anti-oxidant capacity was comparable to that of ascorbyl palmitate, although its antimicrobial activity was weaker than that of benzoic acid [7, 8]. In contrast, ascorbyl cinnamate was of greater interest because cinnamic acid (CA) is a traditional antimicrobial agent [9, 10]. Thomas reported the preparation of specific ascorbyl cinnamate **13–16** via direct esterification, enzymatic esterification or using pre-activated acyl halide and their application as UV filters to protect skin [11]. 6-O-cinnamyl-L-ascorbic acid ester **17** was prepared by reacting

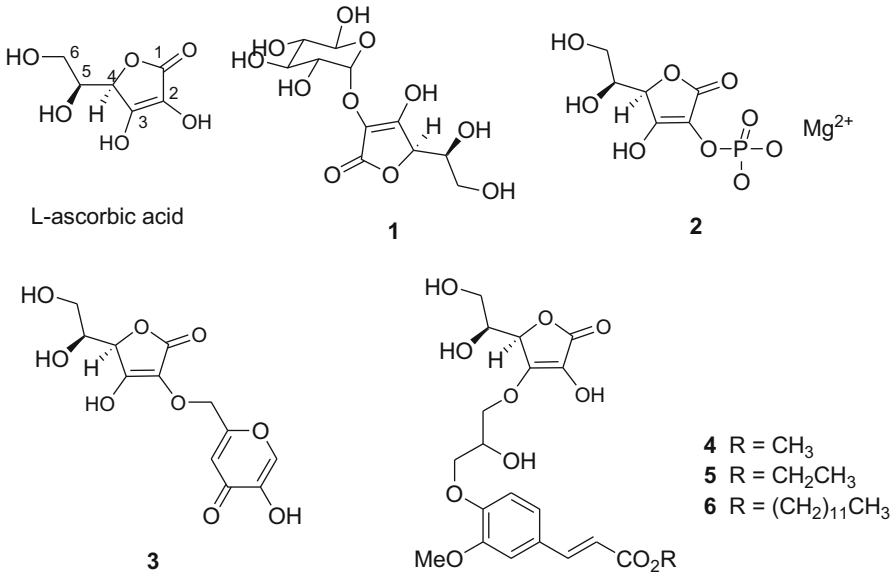


Fig. 1 Ascorbic acid and its novel derivatives modified in the enediol position

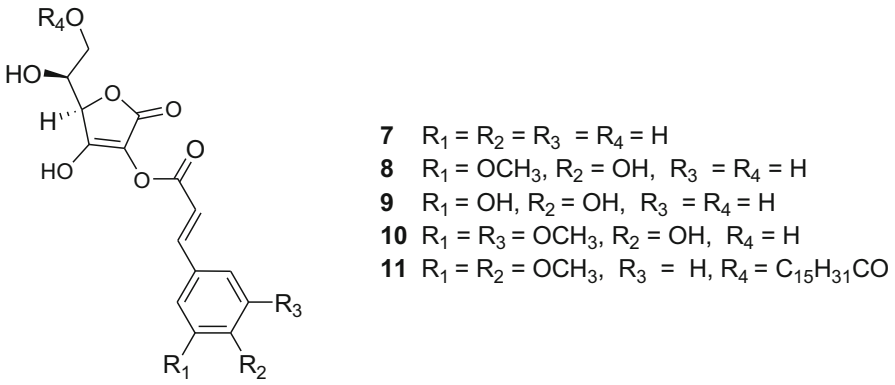


Fig. 2 2-O-ascorbyl cinnamate and its novel derivatives

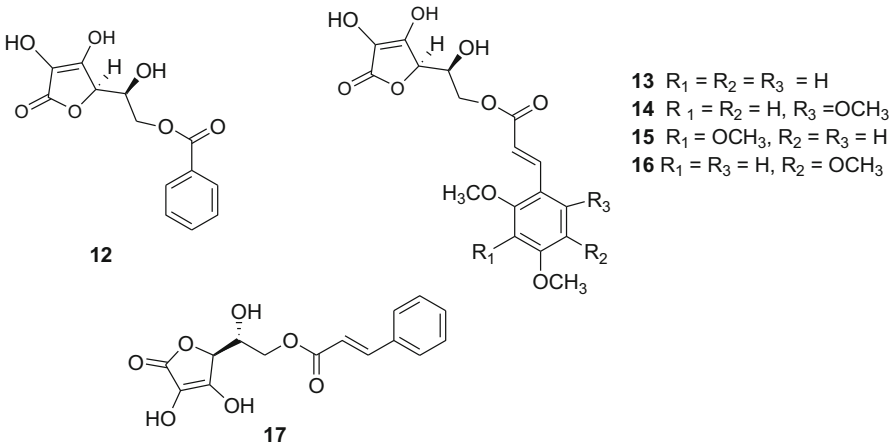
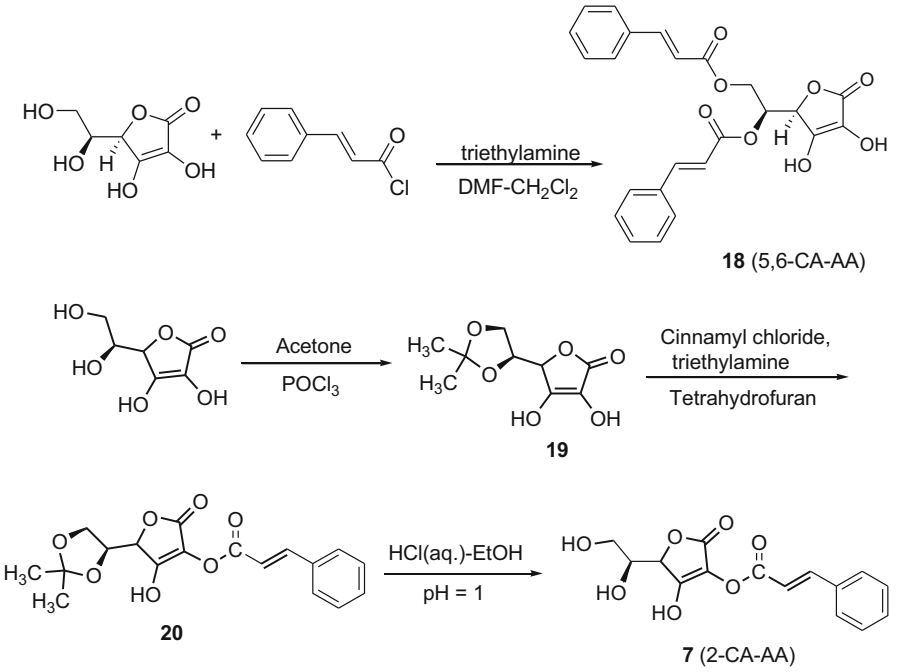


Fig. 3 Some potential bifunctional AA derivatives modified in alcohol position

AA with vinyl cinnamic acid in the presence of immobilized *Candida antarctica* lipase (Novozyme 435), but it only resulted in 68% yield after stirring for 6 days at 30 °C [12]. Shortly afterwards, chemosynthesis of **17** was also realized with 61% yield. However, it required that AA reacted with cinnamyl chloride for 24 h at 0 °C [13].

In the present study, 5,6-di-O-cinnamyl-L-ascorbic acid ester **18** (5,6-CA–AA) was obtained with 76% yield when the reaction was carried out in a mixed solvent of N, N-dimethylformide (DMF) and dichloromethane (CH_2Cl_2) at room temperature (RT). Also, an efficient three-step access to 2-O-cinnamyl-L-ascorbic acid ester **7** (2-CA–AA) with 54% overall yield has been developed (Scheme 1). Moreover, the stability, anti-oxidant and antimicrobial properties of 5,6-CA–AA and 2-CA–AA were further assessed.



Scheme 1 Synthesis of 5,6-di-O-cinnamyl-L-ascorbic acid ester and 2-O-cinnamyl-L-ascorbic acid

Experimental

L-ascorbic acid, cinnamic acid, thionyl chloride, tert-butylhydroquinone (TBHQ), α, α-diphenyl-β-picrylhydrazyl (DPPH) and phenanthroline were purchased from Sinopharm Chemical Reagent (Shanghai, China) or Alfa (Shanghai, China). Potassium sorbate (PS) was purchased from Nantong Acetic Acid Chemical (Nantong, China). All the reagents were used without additional purification. All the solvents and other reagents were of analytical grade. Thin layer chromatography was used to monitor the formation of the products and their chemical structure was determined by infrared spectroscopy (IR), nuclear magnetic resonance (NMR) spectra and electrospray ionization mass spectrometer (ESI–MS). IR spectra were recorded on a Nicolet 5700 FT-IR spectrophotometer. ^{1}H-NMR (600 MHz) and ^{13}C NMR (150 MHz) spectra were recorded on an Agilent VNMRS 600 spectrometer. Chemical shifts are expressed in parts per million downfield from tetramethylsilane as an internal standard. ESI–MS analyses were performed on a Finnigan LCQ Advantage MAX spectrometer.

Escherichia coli ATCC 11775, *Bacillus subtilis* ATCC 6633 and *Staphylococcus aureus* ATCC 6538 were preserved by our laboratory. Nutrient Agar medium was purchased from Hangzhou Microbial Reagent (Hangzhou, China). Refined, bleached and deodorized peanut oil was bought from a local market. Shuanghui chilled pork was bought from Carrefour supermarket in Hefei.

Procedure for synthesis of 5,6-di-O-cinnamyl-L-ascorbic acid (5,6-CA–AA)

Cinnamyl chloride was prepared by reacting cinnamic acid (2.96 g, 20 mmol) with thionyl chloride (12 mL) at 45 °C for 4 h. Then, unreacted thionyl chloride was removed under vacuum and the desired acyl chloride was diluted with 6 mL CH_2Cl_2 and stored on standby. To a solution of AA (1.76 g, 10 mmol) in DMF (8 mL) and CH_2Cl_2 (6 mL) was added triethylamine (Et_3N, 20 mmol) and the mixture was cooled to 0 °C. Cinnamyl chloride (20 mmol, diluted in 6 mL CH_2Cl_2) was added dropwise and the resulting mixture was stirred at RT for 8 h. Water (30 mL) was poured in to terminate the reaction, then extracted with CH_2Cl_2 (30 mL), washed with water, dried with $MgSO_4$ and concentrated in vacuo. The residue was separated by silica gel chromatography with elution consisting of chloroform/methanol (5:1, v/v) to afford 3.08 g of 5,6-di-O-cinnamyl-L-ascorbic acid as a olorless foam solid (yield: 76%). mp 121–123 °C. IR (KBr, cm^{-1}): ν_{max} 3403.6, 3061.1, 3027.9, 2922.8, 2850.8, 1713.6, 1635.8. ^1H NMR (600 MHz, $CDCl_3$): δ 7.33–7.99 (m, 12H, Ph and PhCH=), 6.59–6.62 (m, 1H, =CHCO), 6.48–6.49 (m, 1H, =CHCO), 4.95 (d, 1H, $J = 1.2$ Hz, C4-H), 4.37–4.54 (m, 3H, C5-H, C6-H). ^{13}C NMR (150 MHz, $CDCl_3$): δ 170.1, 166.5, 154.1, 145.2, 135.2, 129.7, 128.1, 126.4, 118.6, 116.9, 74.8, 70.3, 63.1. ESI–MS: m/z 435.12 $[M\text{-}H]^-$.

A small amount of 6-O-cinnamyl-L-ascorbic acid **17** was also obtained during the preparation of 5,6-CA–AA [14]. **17**: ^1H NMR (600 MHz, $CDCl_3$): δ 7.58–7.83 (m, 6H, Ph and PhCH=), 6.42–6.45 (d, 1H, $J = 16.2$ Hz, =CHCO), 4.55–4.59 (m, 3H, C4-H, C6-H), 3.90 (m, 1H, C5-H).

Procedure for synthesis of 2-O-cinnamyl-L-ascorbic acid (2-CA–AA)

To a magnetically stirred solution of AA (1.76 g, 10 mmol) in acetone (9 mL), phosphorus oxychloride (0.5 mL) was added and the reaction mixture was stirred for 4 h at RT. Then it was filtered, washed with cold acetone–water and dried in vacuo, leading to 5,6-O-isopropylidene-L-ascorbic acid **19** being obtained as colorless granules (1.92 g, 89%). IR (KBr, cm^{-1}): ν_{max} 3242.6, 2993.3, 2907.6, 2741.6, 1755.5, 1665.4. ^1H NMR (600 MHz, CD_3COCD_3): δ 4.71–4.73 (m, 1H, C4-H), 4.37–4.39 (m, 1H, C5-H), 4.16–4.21 (m, 1H, C6-H), 3.99–4.03 (m, 1H, C6-H), 1.29 (s, 6H, Me).

The above 5,6-O-isopropylidene-L-ascorbic acid **19** was dissolved in the mixed solvent of tetrahydrofuran (THF, 20 mL) and CH_2Cl_2 (8 mL), and then dried Et_3N (13.5 mmol) was added. The reaction mixture was cooled to 0 °C, cinnamyl chloride (13.5 mmol, 10 mL in CH_2Cl_2) was added dropwise and the resulting solution was stirred at RT for another 6 h. When the reaction was terminated, water (30 mL) and CH_2Cl_2 (30 mL) was added to the mixture, then extracted, washed with water, dried with $MgSO_4$ and concentrated in vacuo. The crude product **20** was obtained without any purification for the next reaction. IR (KBr, cm^{-1}): ν_{max} 3445.6, 3062.6, 2986.9, 2935.6, 1705.0, 1596.6, 1450.7, 1496.9. ^1H NMR (600 MHz, CD_3COCD_3): δ 7.74–7.77 (m, 1H, PhCH=), 7.60–7.66 (m, 2H, Ph), 7.38–7.45 (m, 3H, Ph), 6.56–6.60 (m, 1H, =CHCO), 4.47 (m, 1H,C4-H), 4.33 (m,

1H, C5-H), 4.08–4.11 (m, 1H, C6-H), 4.05–4.06 (m, 1H, C6-H), 1.30 (s, 3H, Me), 1.25 (s, 3H, Me).

The above product **20** was added to a HCl solution (pH 1, 12 mL) and EtOH (8 mL) for de-protection, then the mixture was stirred at RT for 10 h, and evaporated under vacuum, neutralized with $NaHCO_3$ solution, and extracted with ethyl acetate. The organic layer was collected and washed with water, dried with $MgSO_4$ and concentrated in vacuo. The residue was chromatographed on silica gel with elution solvent consisting of chloroform/methanol (5:1, v/v), and 2-O-cinnamyl-L-ascorbic acid **7** (1.65 g, 5.4 mmol, 54% total yield) was obtained as a colorless foam solid. mp 139–141 °C. IR (KBr, cm^{-1}): ν_{max} 3421.1, 1718.0, 3002.0, 2936.3, 1718.0, 1577.2, 1442.1, 1424.7. ^1H NMR (600 MHz, CD_3COCD_3): δ 7.77–7.80 (m, 1H, PhCH=), 7.65–7.71 (m, 2H, Ph), 7.40–7.45 (m, 3H, Ph), 6.64 (d, 1H, $J = 16.2$ Hz, =CHCO), 4.45–4.49 (s, 2H, C5-OH, C6-OH), 4.20–4.27 (d, 1H, $J = 1.2$ Hz, C4-H), 3.95–4.04 (m, 1H, C5-H), 3.70–3.82 (m, 2H, C6-H). ^{13}C NMR (150 MHz, CD_3COCD_3): δ 168.3, 165.3, 158.5, 148.6, 134.9, 128.7, 128.0, 126.5, 115.7, 111.1, 76.8, 68.1, 62.1. ESI–MS: m/z 305.07 $[M-H]^-$.

Stability of 5,6-CA–AA and 2-CA–AA in solution

AA, 5,6-CA–AA (**18**) and 2-CA–AA (**7**) was dissolved in ethanol and scanned by ultraviolet spectrophotometer with full wavelength mode (UV-1700; Ruili, China), and their maximum wavelengths (λ_{max}) were detected at 245, 284, and 265 nm, respectively.

The solutions of AA, 5,6-CA–AA and 2-CA–AA in ethanol were warmed to 37 °C for a period of time. The absorbance at λ_{max} was measured at intervals of 1 h. Then, the solutions of 5,6-CA–AA and 2-CA–AA in ethanol-phosphate buffer solution (PBS) were stored at RT for several weeks, and the absorbance was similarly recorded.

Anti-oxidant property of 5,6-CA–AA and 2-CA–AA

DPPH radical scavenging assay

The scavenging activity (SA) of AA, 5,6-CA–AA, 2-CA–AA and TBHQ against DPPH radical were assessed as described in the previous method [15]. Briefly, DPPH (0.2 mM, 2 mL) was mixed with different concentrations of the tested compounds (20–100 μM in EtOH, 2 mL) and the reaction was carried out at 25 °C for 30 min. Then, the absorbance at 517 nm was denoted as A_1. Similarly, ethanol (2 mL) was used to replace the sample as the control. SA was measured according to the following formula.

$$\text{SA } (\%) = \frac{(A_0 - A_1)}{A_0} \times 100\%$$

where A_1, A_0 are the absorbance of the sample and control, respectively.

Hydroxyl radical scavenging assay

The SA of AA, 5,6-CA–AA, 2-CA–AA and TBHQ against hydroxyl-free radicals were assessed by a modified Fenton reaction protocol [16]. Briefly, 1,10-phenanthroline (0.75 mM in ethanol, 1 mL) and PBS (pH 7.4, 1 mL) was mixed with $FeSO_4$ solution (0.75 mM, 1 mL) and the tested compound (2–10 mM in EtOH, 1 mL), H_2O_2 solution (0.01%, 1 mL) was added to the above mixture and the reaction was carried out at 37 °C for 1 h. Deionized water (1 mL) was used to replace H_2O_2 as the blank (A_{00}) and 2 mL deionized water was used to replace H_2O_2 and the sample as the control (A_0). The SA of the tested compound was evaluated by the following formula:

$$\text{SA } (\%) = \frac{(A_1 - A_{00})}{(A_0 - A_{00})} \times 100\%$$

where A_1, A_0, and A_{00} are the absorbance of the sample, control and blank, respectively.

Antilipid-oxidation capacity of ascorbyl cinnamate in peanut oil

In the experiment, the modified Schaal oven method was adapted to accelerate the oxidation of the peanut oil at 100 ± 1 °C [7]. The above 5,6-CA–AA, 2-CA–AA and TBHQ were added to the peanut oil (50 g) at a dose of 0.05%, and the sample without any special compound was made the control. All the samples were placed in an oven at 100 °C for 2, 4, 6, 8, 10, 12, 14 and 16 h, respectively. Then, their respective peroxide values (PV) were measured by iodometric determination [17] and represented by the average of triplicate samples.

Antimicrobial activity screening

Antibacterial activity using disc diffusion method

The inhibitory effect of 5,6-CA–AA, 2-CA–AA, CA and PS on various microorganisms was determined according to the filter paper disc diffusion method [18, 19]. *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* were activated in nutrient agar at 37 °C for 24 h, then diluted 10^8 cfu/mL with sterile physiological saline and shocked uniformly. Under aseptic operation, 1 mL bacterial suspension was coated on a plate. Then, 10 mL medium was added to make it uniform on standby. Next, a 6-mm sterilized paper disc was loaded onto the surface of the plate, and the control (DMF and sterile saline, denoted as CK1 and CK2, respectively) or 20 μL diluted compounds [40, 20, 10, 5, and 2.5 mg/mL in DMF (5,6-CA–AA, 2-CA–AA and CA) or saline (PS)] were added dropwise on paper disc. Thus, 800, 400, 200, 100,and 50 μg test compounds were absorbed on the disc and the ultimate plate was incubated at 37 °C for 24 h. Finally, the zone of inhibition (mm) was measured.

Dual inhibition of ascorbyl cinnamate on microbial growth and lipid oxidation

The antimicrobial and antilipid oxidation effect of ascorbyl cinnamate was assayed by the "pork-by-soaking" method. The test solution was prepared from the mixture of 5,6-CA–AA and 2-CA–AA (1:1, m/m), CA or PS dissolved in 1% acetic acid aqueous solution, and the added amount of reagent was 0.05% (m/v). Each 25 g of pork hind leg was soaked in the tested solution (50 mL) and the control sample was treated with 1% acetic acid aqueous solution for 10 min. All the treated pork was drained, sealed with plastic wrap and stored in a refrigerator at 4 °C for a period of time. Total numbers of colonies [20] and thiobarbituric acid reactive substance (TBARS) content [21] were analyzed after 0, 3, 5, 7, and 9 days.

Statistical analysis

Analysis of variance (ANOVA) was carried out using SPSS 19.0 software to test for any significant differences between the groups. Differences between groups at 5% ($P < 0.05$) were considered significant.

Results and discussion

Synthesis of ascorbyl cinnamate

First, the reaction of AA with cinnamyl chloride was performed in CH_2Cl_2 at RT and the conversion was very poor, which was ascribed to the poor solubility of AA in this solvent. Therefore, DMF was introduced and the reaction was clearly enhanced. The major product was obtained with 65% isolated yield when it was carried out in DMF-CH_2Cl_2 (1:3, v/v) and the molar ratio of AA, cinnamyl chloride and Et_3N was 1:2.0:2.0 (Scheme 1). Next, this product was identified as 5,6-CA–AA, which was different from that of the in lipase catalysis or chemosynthesis in dimethylacetamide (DMAc)-CH_2Cl_2 [12, 13]. They both gave **17** as the major product instead of 5,6-CA–AA. It should be noted that **17** was also detected as a by-product in the above synthetic procedure [14].

Subsequently, the influence of cinnamyl chloride dose, substrate concentration and reaction temperature on the preparation of 5,6-CA–AA was investigated. Unfortunately, these efforts did not bring evident gains. Finally, 5,6-CA–AA was still the main product of the reaction and 76% yield was achieved in DMF-CH_2Cl_2 (2:3, v/v).

Similarly, the synthesis procedure of 2-CA–AA (**7**, Scheme 1) was also studied. Among the three steps, the second esterified product **20** was gained with better yield when it was performed in THF/CH_2Cl_2 and the molar ratio of 5,6-O-isopropylidene-L-ascorbic acid **19**, cinnamyl chloride and Et_3N was 1:1.5:1.5. Then, compound **20** was directly de-protected under the optimal pH of 1, resulting in 2-CA–AA with 54% total yield. This result was better than that reported by Galey [6], which gave only 32.6% overall yield. By comparison, our present synthetic procedure was more efficient, increasing the output of 2-CA–AA by 65.6%.

Stability of ascorbyl cinnamate in solution

The absorbance of AA, 5,6-CA–AA and 2-CA–AA at λ_{max} during storage is demonstrated in Fig. 4. With the extension of the storage period, the absorbance of AA decreased from the initial 0.661 to 0.103, which was consistent with that of AA instability in solution. As for ascorbyl cinnamate, they were both relatively stable. Thus, a further stability assay was conducted in EtOH-PBS. Within 30 days, almost no significant changes were observed, their RSD of absorbance being only 1.58 and 0.95%, respectively, as shown in Table 1. The above results indicated that the stability of 5,6-CA–AA and 2-CA–AA was greatly improved.

In vitro anti-oxidant activity of ascorbyl cinnamate

The DPPH and hydroxyl radical scavenging assay are two of the most common methods for assessing anti-oxidant activities [22]. The above synthesized compounds were evaluated as anti-oxidant agents in comparing to TBHQ, a perfect anti-oxidant in lipid system. The anti-oxidant activity of each compound was expressed in terms of EC_{50} (the effective molar concentration required to scavenge 50% of DPPH or hyrdorxyl radical). The SA of AA, 5,6-CA–AA, 2-CA–AA and TBHQ against DPPH or hydroxyl radical were measured and the corresponding EC_{50} values are shown in Table 2. In the DPPH radical scavenging test, 5,6-CA–AA almost maintained the inherent scavenging ability of AA (EC_{50}: 63.96 vs. 59.78 μM) even though ascorbyl cinnamate exhibited poorer capacity than that of TBHQ (EC_{50}: 46.31 μM). Also, it was noted that the EC_{50} of AA (59.78 μM, 10.5 μg/mL) under this condition was not entirely in accordance with the data reported in the literature [23–25], which gave 55, 39.81,and 5.58 μg/mL respectively. The discrepancy was likely because of different concentration ranges, sampling times and investigators. However, a delightful fact was observed in the hydroxyl radical scavenging assay in that 5,6-CA–AA was superior to that of TBHQ

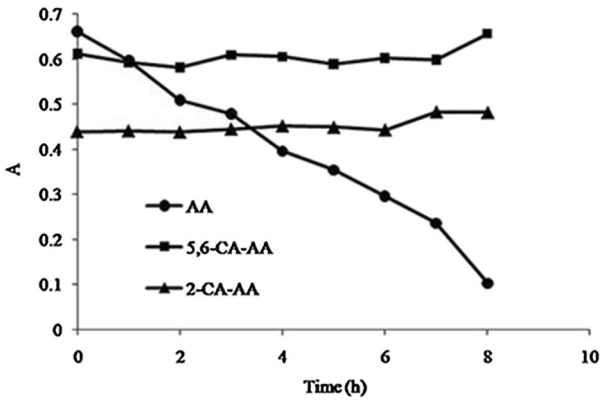


Fig. 4 Effect of storage time on the absorbance of AA and ascorbyl cinnamate

Table 1 Effect of storage time on the absorbance of ascorbyl cinnamate

Time (d)	5,6-CA–AA	RSD (%)	2-CA–AA	RSD (%)
1	0.563		0.483	
5	0.571		0.481	
10	0.582		0.485	
15	0.577	1.58	0.479	0.95
20	0.558		0.491	
25	0.562		0.478	
30	0.576		0.487	

Table 2 EC_{50} for DPPH and hydroxyl inhibition of chemical compounds

Compound	EC_{50} for DPPH (μM)	EC_{50} for hydroxyl radical (mM)
5,6-CA–AA	63.96 ± 0.18	7.84 ± 0.05
2-CA–AA	81.19 ± 0.21	9.72 ± 0.03
AA	59.78 ± 0.09	5.78 ± 0.05
TBHQ	46.31 ± 0.10	8.38 ± 0.09

(EC_{50}: 7.84 vs. 8.38 mM). If a comparison was made between ascorbyl cinnamate and AA, ascorbyl cinnamate exhibited poorer capacity than that of AA (EC_{50}: 5.78 mM), which was similar to the previous report of ascorbyl fatty acid esters [26]. In a word, a steady trend was that 5,6-CA–AA showed better anti-oxidant capacity than that of 2-CA–AA whether in the DPPH or hydroxyl radical scavenging tesst, which was consistent with that of vitamin C phosphodiesters linked at the C-2 hydroxyl group with lower anti-oxidant activity [3].

Ascorbyl cinnamate exhibited relative stability in solution and medium anti-oxidant activity in the above chemical system. Subsequently, it was added to an oil-soluble system for its antilipid-oxidation evaluation. Peroxide value (PV) is an indicator to control oily feed, food quality and safety, a lower PV in lipid oxidation means better anti-oxidant activity of a substrate. Thus, ascorbyl cinnamate, TBHQ, was added to peanut oil and stored at an elevated 100 °C. As the results show in Fig. 5, the hydroperoxides formation was remarkably retarded by the compounds such as 5,6-CA–AA, 2-CA–AA and TBHQ, especially in the duration of 10–16 h ($P < 0.01$, any one vs. control). When the duration reached 16 h, the corresponding PVs level decreased from an initial 90.13 meq/kg to 61.27, 68.86, and 43.04 meq/kg, respectively. As seen from the data, TBHQ gave the outstanding anti-oxidation with 52.2% inhibition. Next, a decrease of 32.0% in PV was obtained in that of 5,6-CA–AA. On the other hand, there was no significant difference in the anti-oxidant effect between the two derivatives of AA during the first duration of 8 h ($P > 0.05$, 5,6-CA–AA vs. 2-CA–AA). But with the lipid oxidation continued, 5,6-CA–AA showed stronger anti-oxidant capacity than that of 2-CA–AA ($P < 0.01$, 5,6-CA–

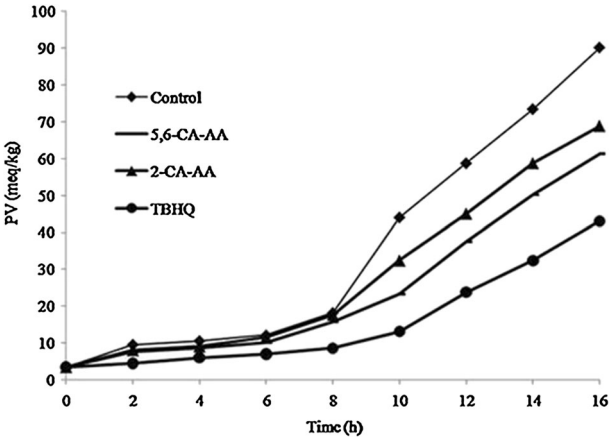


Fig. 5 PVs of peanut oil in the presence of ascorbyl cinnamate at 100 °C

AA vs. 2-CA–AA, maintained for 10–16 h), which was in line with the sequence of the DPPH and hydroxyl radical scavenging assays above mentioned.

Antimicrobial activity of ascorbyl cinnamate

Since CA has antibacterial activity [9, 10], the inherent property was likely to be transplanted into these two ascorbyl cinnamate. Therefore, the inhibition of 5,6-CA–AA and 2-CA–AA against *Escherichia coli*, *Bacillus subtilis* and *Staphylococcus aureus* were assessed, using CA as a reference and PS as standard antimicrobial agent. PS, the water-soluble salts of sorbic acid, is an effective and widespread preservative used in many foods such as meat, drink and cheese [27, 28]. The antimicrobial results are represented as the zone of inhibition (mm) in Table 3. Overall, the four test compounds had demonstrated medium to good antimicrobial activity against both Gram-positive and Gram-negative bacteria and the effect was dose-dependent. Among them, the antimicrobial ability of PS was the most prominent no matter what type of microbes. Another obvious trend was that the effect of 5,6-CA–AA or 2-CA–AA was not less than that of CA when the absorptive amount ranged from 200 to 800 μg, which showed that the inherent antimicrobial property of CA was well migrated to the AA derivatives. However, there were some differences in the performance of the two AA derivatives: 2-CA–AA possessed a relatively weaker antimicrobial ability than that of 5,6-CA–AA in the assays of *Escherichia coli* or *Bacillus subtilis*, which did not affect its potential application as an antimicrobial agent since esters had a wide range of active pH over the acids range [29].

In view of the fact that the two AA derivatives had medium antibacterial activity against exogenous strains and antilipid oxidation in peanut oil, they may be potential bifunctional compounds inhibiting microbial growth and fat oxidation. Based on this consideration, pork was chosen as a suitable system to evaluate the dual effect of the ascorbyl cinnamate. Meanwhile, PS is also considered as a

Table 3 Inhibition zone of 5,6-CA–AA, 2-CA–AA and CA (mm)

Species	Compound	Absorptive amount (μg)					Control	
		800	400	200	100	50	CK_1	CK_2
Escherichia coli	5,6-CA–AA	16	13	12	8 (±)	–	–	–
	2-CA–AA	15	12 (±)	9	8 (±)	–	–	–
	CA	11	9	8 (±)	–	–	–	–
	PS	24	19	17	13	8 (±)	–	–
Bacillus subtilis	5,6-CA–AA	18 (±)	17	16	11	–	–	–
	2-CA–AA	16	14	11(±)	–	–	–	–
	CA	12	11	10	9	–	–	–
	PS	22	20	15	13	10 (±)	–	–
Staphylococcus aureus	5,6-CA–AA	15	12	8 (±)	–	–	–	–
	2-CA–AA	16	12	9 (±)	–	–	–	–
	CA	12	10	9 (±)	8 (±)	–	–	–
	PS	23	20	17	13	10 (±)	–	–

± Antimicrobial ring is fuzzy; – no antimicrobial ring

positive referent compound because it has been listed as a preservative and antioxidant in the "National Food Safety Standard for Uses of Food Additives" (GB 2760-2014) [30]. TBARS content is widely used as an indicator of the degree of lipid oxidation. In general, people can feel the peculiar smell once the TBARS is more than 0.5 mg/kg [31]. Therefore, the total number of colonies and TBARS content of the pork treated with ascorbyl cinnamate, CA, and PS was measured on different storage days, as shown in Table 4. It was clear that the microbial reproduction of the pork had slowed down after soaking in any solution of the test compounds. Among the three compounds, the inhibitory effects induced by ascorbyl cinnamate or PS were significant during the storage ($P < 0.01$, mixed ascorbyl cinnamate vs. control; $P < 0.01$, PS vs. control). In contrast, the inhibition of CA was relatively obvious only in the storage of 5–9 days ($P < 0.01$, $P < 0.05$, CA vs. control). A similar phenomenon was found in the result of TBARS content. The use of any special compound greatly retarded the TBARS level of the pork [$P < 0.01$, ascorbyl cinnamate (PS) vs. control; $P < 0.05$, $P < 0.01$, CA vs. Control]. Moreover, the sample treated with mixed ascorbyl cinnamate had a lower TBARS content than that of PS during the storage, but the difference was significant only on the fifth or ninth day (0.20 ± 0.03 vs. 0.28 ± 0.01, $P < 0.05$; 0.45 ± 0.02 vs. 0.54 ± 0.06, $P < 0.05$). Taking the TBARS level of the fifth day as an example, a decline of up to 56.5% was found in the pork treated with ascorbyl cinnamate. Under the same conditions, the application of PS only gave a 39.1% reduction compared with control.These results indicate that ascorbyl cinnamate exhibited a promising dual inhibition on the lipid oxidation and microbial reproduction although its preservative effect on pork was slightly weaker than that of the positive referent compound ($P < 0.05$, ascorbyl cinnamate vs. PS, on the fifth, seventh, and

Table 4 Total bacterial, TBARS content of pork in the presence of ascorbyl cinnamate

Item	Compound	Storage (d)				
		0	3	5	7	9
Total bacterial (lgcfu g^{-1})	Control	2.58 ± 0.21	4.67 ± 0.20	6.63 ± 0.25	7.50 ± 0.41	8.01 ± 0.39
	Ascorbyl cinnamate	2.61 ± 0.30	3.10 ± 0.19[b]	4.18 ± 0.18[b,c]	5.21 ± 0.28[b,c]	6.03 ± 0.20[b,c]
	CA	2.50 ± 0.14	3.70 ± 0.21	4.70 ± 0.17[a]	5.49 ± 0.18[b]	6.75 ± 0.28[a]
	PS	2.57 ± 0.12	3.02 ± 0.10[b]	3.58 ± 0.12[b]	4.12 ± 0.21[b]	4.84 ± 0.24[b]
TBARS (mg/kg)	Control	0.13 ± 0.02	0.25 ± 0.03	0.46 ± 0.03	0.57 ± 0.06	0.67 ± 0.05
	Ascorbyl cinnamate	0.13 ± 0.01	0.13 ± 0.02[b]	0.20 ± 0.03[b,c]	0.35 ± 0.03[b]	0.45 ± 0.02[b,c]
	CA	0.12 ± 0.01	0.20 ± 0.02[a]	0.29 ± 0.01[b]	0.45 ± 0.02[b]	0.60 ± 0.09[a]
	PS	0.13 ± 0.01	0.15 ± 0.01[b]	0.28 ± 0.01[b]	0.39 ± 0.02[b]	0.54 ± 0.06[b]

[a] $P < 0.05$, compared with control

[b] $P < 0.01$ compared with control

[c] $P < 0.05$, ascorbyl cinnamate versus PS

ninth days). Also, the derivative modified in the hydroxyl position of AA such as 5,6-CA–AA may be a potential bifunctional candidate in future food, cosmetics or drug trials.

Conclusion

Two novel ascorbyl cinnamates, 5,6-CA–AA and 2-CA–AA, were successfully prepared with 76 and 54% yields, respectively. Of the two, 5,6-CA–AA was first synthesized and an efficient access to 2-CA–AA was developed with a 65.6% increase in the yield. The subsequent investigation indicated that 5,6-CA–AA maintained the inherent anti-oxidant function of AA. Also, 5,6-CA–AA and 2-CA–AA possessed antimicrobial activity as well as CA in vitro. The application of ascorbyl cinnamate could effectively retard the lipid oxidation of peanut oil or pork, and the microbial reproduction of pork was inhibited to some extent. This work offers an excellent approach for the derivation of AA, which may lead to the discovery of potent efficient bifunctional compounds.

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