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Esterification of the Free Carboxylic Group in 3,4-di-O-caffeoylquinic Acid Enhances the Inhibition Activity against Respiratory Syncytial Virus (RSV)

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Abstract

Based on molecular docking analysis, nine alkyl 3,4-di-*O*-caffeoyl- quinates have been designed. Started from 3,4-di-*O*-caffeoylquinic acid (3,4-DCQA), all the compounds have been synthesized using thionylchloride as coupling reagent with yields of 56%-72%. By applying plaque reduction assay, the anti-respiratory syncytial virus (RSV) activities of all the compounds were evaluated. The IC_{50} values of all the derivatives were 2.9 to 7.8 times less than that of 3,4-DCQA itself. Isopropyl 3,4-Di-*O*-caffeoyl quinate (LS-4) was confirmed as the most active compound with IC_{50} value of 0.3 μM. It was indicated that hydrophobic groups with 2 to 4 carbon chain length were optimal for the enhancement of anti-RSV activity. Interestingly, all the derivatives showed greater cytotoxicity than 3,4-DCQA. Except LS-5, LS-6 and LS-9, all the derivatives have less toxicity than ribavirin. Furthermore, the stabilities of LS-4 in water, artificial gastric juice (AGJ), and artificial intestinal juice were evaluated, respectively. It was shown that LS-4 is stable in AGJ with the hydrolysis rate of 24.9% after 6 hours incubation at 37°C.

Keywords: Caffeoylquinic acid; RSV inhibitor; RSV fusion protein; Molecular docking

Introduction

Human respiratory syncytial virus (RSV) is a member of the Paramyxoviradae. Exposure to RSV is essentially universal for those under 2 years of age and re-infection is common, a consequence of an incomplete immune response of limited durability. Based on serological evidence, approximately 95% of children have been exposed to RSV by 2 years of age, and 100% of children have been exposed by the time they reach adulthood [1]. Because the immune response to RSV infection is not protective, RSV infections may re-occur throughout adulthood [2], and can also occur as a co-infection with other respiratory pathogens, leading to exacerbated symptoms [3,4]. It is noteworthy that infants with underlying cardiopulmonary problems, the elderly, and the immuno-compromised adults are the most vulnerable to serious complications or even at risk for death from RSV infection [1,5,6]. Global mortality rates in children under five years of age have been estimated at 200,000 annually [7]. Of particular note, high levels of mortality are associated with outbreaks of RSV in bone marrow transplant units [8,9].

Many efforts have been carried on to find the effective treatment or prevention of RSV infections [10-13]. However, currently, the treatment of RSV infection is limited to administration of the nucleoside analogue ribavirin (Figure 1), a teratogen with an enigmatic mode of action that is frequently administered as an aerosol, which is very long and inconvenient regimen required for its delivery [14]. Also, the utilization of ribavirin is limited due to its efficacy and toxicity concerns [15,16]. As a matter of fact, there are no approved vaccines, the only prophylactic therapies available are RSVIGIV, a polyclonal RSV immunoglobulin, and Synagis (palivizumab), a human monoclonal antibody targeting the RSV fusion protein [17-19]. The cost of using palivizumab is quite expensive [20-22]. This restricts its widespread use. Therfore, the need for potent, selective, and efficacious inhibitors of RSV suitable for clinical use remains quite urgent.

As an on-going progress to develop anti-infection agents, 3,4-di-*O*-caffeoylquinic acid (3,4-DCQA) and 3,5-di-*O*-caffeoylquinic acid (3,5-DCQA) (Figure 1) caught our eyes. These two compounds were identified from *Youngia japonica*, and *Schefflera heptaphylla*, respectively [23,24], which are two traditional Chinese medicinal plants used for the treatment of infections. 3,4-DCQA and 3,5-DCQA were confirmed with specific anti-RSV activity [24], which showed better activity than ribavirin. Both compounds had no obvious antiviral activity against influenza A (Flu A), Coxsackie B3 (Cox B3), and Herpes simplex type one (HSV-1) viruses. More interestingly, they exhibited minimal cytotoxicity against HEp-2 cells with median cytotoxic concentration (CC_{50}) higher than 1000 μM and the maximal non-cytotoxic concentration (MNCC) were about 96.7 μM, which indicated that their anti-RSV effect was not due to cytotoxicity. Further investigations showed that 3,4-DCQA is an effective inhibitor of RSV fusion protein (RSV-F) [25]. It seems to us that 3,4-DCQA is a good lead compound for the development of anti-RSV agents.

Nowadays, it is recognized that RSV-F is a protein that mediates the fusion of viral envelope with host cell membrane and is regarded as a prominent target for therapeutic intervention [26-28]. A previous molecular docking study showed that 3,4-DCQA binds RSV-F well. Interestingly, it was found that there is a hydrophobic pocket formed by B/Tyr87, B/Ala43, B/Pro40 and A/Tyr91 that is closed to 1-carboxylic group (Figure 2). We predicted that hydrophobic extension (R group) of 1-carboxylic group in 3,4-DCQA to this pocket may enhance the binding affinity and selectivity.

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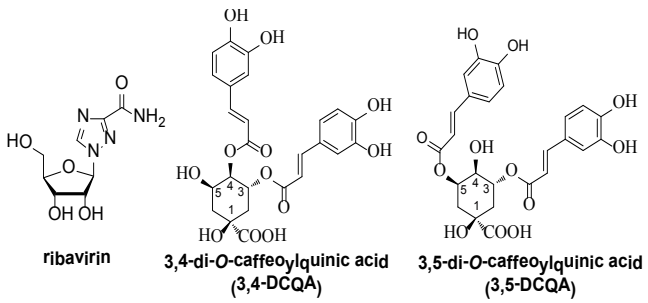


Figure 1: Chemical structures of ribavirin and caffeoylquinic acids.

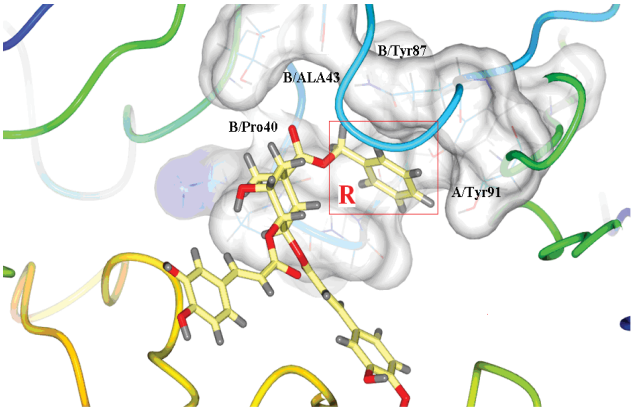


Figure 2: Complex model of 3,4-DCQ-1-COOR with respiratory syncytial virus (RSV) fusion protein (PDB: 3IXT). R (within the red rectangle) represents any hydrophobic group. R is buried in a hydrophobic pocket formed by B/TYR87, B/ALA43, B/PRO40, and A/TYR91. B means basic. A means acidic.

In order to explore the effects of R group, in the current study, a series of alkyl 3,4-di-*O*-caffeoylquinates have been designed and synthesized. The systematic investigation of their RSV inhibition, structure-activity relationship, molecular docking analysis and stabilities were also reported here.

Experimental Section

Materials and methods

Materials: Isochlorogenic acid B was purchased from Shanghai YiFei Biotechnology Company (China). All other chemicals were purchased from Aldrich or Adamas without further purification. Silica gel for column chromatography was purchased from Qingdao Marine Chemicals Inc., China. Chromatographic grade methanol acetonitrile was bought from MREDA Technology Inc. (USA).

General experimental procedures: NMR spectra were recorded on Bruker AV-300 (Bruker Biospin, Swiss), TMS was used as internal standard. ESI-MS were recorded on Finnigan LCQ Advantage MAX mass spectrometer. HPLC was performed on either LC-100 liquid chromatograph equipped with a tunable LC-100 UV detector (Shanghai Wufeng Inc., China) or Agilent 1200 series liquid chromatograph equipped with an Agilent 1200 Series UV detector (Agilent Technologies, USA). Column used were Cosmosil 5C_{18} (Nacalai Tesque Inc., Japan) for general purification. Pre-coated thin-layer chromatography (TLC) plates (Institute of Yantai Chemical Industry, China) were used for TLC. Spots on TLC plates were detected by either a ZF-7A portable UV detector or spraying Bismuth potassium iodide solution followed subsequent heating. Ethanol was reluxed over Fresh magnesium ribbon for 5 hours and redistilled.

Molecular docking: The 3,4-DCQA and its derivatives were sketched in SYBYL 8.1 (Tripos, Inc., St. Louis, MO, USA) molecular modeling package and Gasteiger-Hückel charges were assigned to the atoms of all the compounds. Then they were docked to RSV-F protein (PDB entry: 3IXT) using Glide program [29] in its XP mode in a standard procedure. The three water molecules at the active site were all retained. The docked conformations of the compounds with the lowest energy were selected for further analysis.

Stability: Preparation of artificial gastric juice (AGJ): NaCl (2.0 g) and pepsin (3.2 g) were dissolved in an appropriate amount of water. Then concentrated hydrochloride (7.0 ml) was added under stirring. The mixture was diluted with water to a constant volume of 1000 ml.

Preparation of artificial intestinal juice (AIJ): KH_2PO_4 (6.8 g) and trypsin (10.0 g) were dissolved in an appropriate amount of water. Then the mixture was diluted with water to a constant volume of 1000 ml. The pH value of the solution was adjusted to 7.5 ± 0.1 with 0.4% NaOH solution.

Standard concentration curve for LS-4: Solutions with exact concentrations of 1.0, 0.25, 0.125, 0.03125, and 0.015063 mg/ml were carefully prepared. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Cosmosil column (C_{18}, 5 Å, 4.6 × 250 mm) using an LC-100 fluid unit (Shanghai Wufeng Inc., China) with a tunable LC-100 UV detector. 10 µl of each solution was injected, and the HPLC profile was obtained with eluant of 30 vol% acetonitril against double-distilled water under UV detection (330 nm). The retension time was 14.3 min. Peak area under the curve was plotted against concentration. The experiments were repeated at least three times and compared with the control. The equation obtained was $y=8 \times 10^6 x+48569$, and the $R^2=0.9999$.

Compound LS-4 was incubated in artificial gastric juice (AGJ), artificial intestinal juice (AIJ), and double-distilled water at 37°C, respectively, for a period of 6 h. Analyses of samples were scheduled at 0, 2.0, 4.0, and 6.0 h, respectively, using RP-HPLC method as described above. At each time point, the concentration ($[C]_t$) was determined by standard concentration curve. The experiments were repeated at least three times and compared with the control.

The hydrolysis rate was calculated as following:

$$rate(\%) = \frac{[C]_t - [C]_0}{[C]_0} \times 100\%$$

Synthetic process

Synthesis of methyl 3,5-di-*O*-caffeoylquinate (LS-1): Isochlorogenic acid B (0.1 g, 0.19 mmol) was dissolved in 5 mL of dried methanol and kept at -15°C for 5 min, then thionylchloride (0.03 ml, 0.38 mmol) was added dropwise with stirring. After 30 min, the reaction mixture was transferred to room temperature and stirred overnight. Then 3 ml of water was added slowly to dispose the excess thionylchloride in ice-water bath. The reaction mixture was extracted with EtOAc (4 × 100 mL). The combined organic layers were washed with water and brine, respectively, and were dried over anhydrous Na_2SO_4. After removal of the solvent, the residue was purified by RP-HPLC (acetonitrile:H_2O=3:7, *V:V*) to give LS-1 as slight yellow powder (58.2 mg, 56.7%). ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.58 (d, *J*=12.0 Hz, 1H, H-7'), 7.53 (d, *J*=12.0 Hz, 1H, H-7''), 7.15 (m, 2H, H-2', 2''), 7.00 (m, 2H, H-6', 6''), 6.86 (m, 2H, H-5', 5''), 6.31 (d, *J*=12.0 Hz, 1H, H-8'), 6.26 (d, *J*=12.0 Hz, 1H, H-8''), 5.60 (m, 1H, H-3), 5.06 (dd, *J*=6.3, 2.4 Hz, 1H, H-4), 4.39 (m, 1H, H-5), 3.71 (s, 3H, OCH_3), 2.10-2.41 (m, 4H, H-2, 6); ^{13}C NMR (75 MHz, acetone-d_6) δ: 175.4, 167.0, 166.8, 148.9,

148.8, 146.3 (2C), 146.1, 146.0, 127.6, 127.5, 122.7, 122.6, 116.4 (2C), 115.8, 115.5, 115.2 (2C), 74.8, 74.6, 68.4, 65.1, 52.6, 41.1, 36.7; ESI-MS (*m/z*): 529.5[M-H]$^-$.

Synthesis of ethyl 3,5-di-*O*-caffeoylquinate (LS-2): Followed the process as described above. Isochlorogenic acid B (0.1 g, 0.19 mmol), thionylchloride (0.03 ml, 0.38 mmol), 5 mL of dried ethanol were added. The final purification by RP-HPLC (acetonitrile:H_2O=3:7, *V:V*) gave LS-2 as brown powder (72.4 mg, 68.7%). ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.56 (d, *J*=12.0 Hz, 1H, H-7'), 7.51 (d, *J*=12.0 Hz, 1H, H-7''), 7.14 (m, 2H, H-2', 2''), 6.98 (m, 2H, H-6', 6''), 6.85 (m, 2H, H-5', 5''), 6.28 (d, *J*=12.0 Hz, 1H, H-8'), 6.23 (d, *J*=12.0 Hz, 1H, H-8''), 5.58 (m, 1H, H-3), 5.06 (dd, *J*=6.3, 2.4 Hz, 1H, H-4), 4.36 (m, 1H, H-5), 4.16 (q, *J*=6.0 Hz, 2H, H-8), 2.07-2.37 (m, 4H, H-2, 6), 1.25 (t, *J*=6.0 Hz, 3H, H-9); ^{13}C NMR (75 MHz, acetone-d_6) δ: 175.0, 167.1, 166.8, 149.0, 148.9, 146.4 (2C), 146.2, 146.0, 127.6, 127.5, 122.7, 122.6, 116.4 (2C), 115.8, 115.5, 115.2 (2C), 74.9, 74.5, 69.4, 65.9, 61.7, 41.2, 36.7, 14.4; ESI-MS (*m/z*): 543.9[M-H]$^-$.

Synthesis of *n*-propyl 3,5-di-*O*-caffeoylquinate (LS-3): Followed the process as described above. Isochlorogenic acid B (0.1 g, 0.19 mmol), thionylchloride (0.03 ml, 0.38 mmol), 5 mL of dried *n*-propanol were added. The final purification by RP-HPLC (acetonitrile:H_2O=7:13, *V:V*) to give LS-3 as slight yellow powder (73.0 mg, 67.5%). ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.57 (d, *J*=12.0 Hz, 1H, H-7'), 7.52 (d, *J*=12.0 Hz, 1H, H-7''), 7.15 (m, 2H, H-2', 2''), 7.00 (m, 2H, H-6', 6''), 6.85 (m, 2H, H-5', 5''), 6.30 (d, *J*=12.0 Hz, 1H, H-8'), 6.24 (d, *J*=12.0 Hz, 1H, H-8''), 5.60 (m, 1H, H-3), 5.08 (dd, *J*=6.3, 2.4 Hz, 1H, H-4), 4.38 (m, 1H, H-5), 4.09 (t, *J*=6.0 Hz, 2H, H-8), 2.09-2.40 (m, 4H, H-2, 6), 1.68 (sext, *J*=6.0 Hz, 2H, H-9), 0.96 (t, *J*=6.0 Hz, 3H, H-10); ^{13}C NMR (75 MHz, acetone-d_6) δ: 175.1, 167.0, 166.8, 148.9, 148.8, 146.4 (2C), 146.1, 146.0, 127.7, 127.6, 122.7, 122.6, 116.5 (2C), 115.9, 115.6, 115.3 (2C), 75.0, 74.7, 69.4, 67.3, 66.0, 41.4, 36.8, 22.7, 10.8; ESI-MS (*m/z*): 581.5[M+Na]$^+$, 1139.1[2M+Na]$^+$.

Synthesis of iso-propyl 3,5-di-*O*-caffeoylquinate (LS-4): Followed the process as described above. Isochlorogenic acid B (0.1 g, 0.19 mmol), thionylchloride (0.03 ml, 0.38 mmol), 5 mL of dried *i*-propanol were added. The final purification by RP-HPLC (acetonitrile:H_2O=7:13, *V:V*) to give LS-4 as yellow oil (71.7 mg, 66.3%). $[\alpha]_D^{25}$ -56.2°(c=1.0, MeOH); ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.57 (d, *J*=12.0 Hz, 1H, H-7'), 7.52 (d, *J*=12.0 Hz, 1H, H-7''), 7.15 (m, 2H, H-2', 2''), 6.98 (m, 2H, H-6', 6''), 6.86 (m, 2H, H-5', 5''), 6.30 (d, *J*=12.0 Hz, 1H, H-8'), 6.24 (d, *J*=12.0 Hz, 1H, H-8''), 5.59 (m, 1H, H-3), 5.07 (dd, *J*=6.3, 2.4 Hz, 1H, H-4), 4.99 (heptet, *J*=6.0 Hz, 1H, H-8), 4.37 (m, 1H, H-5), 2.07-2.38 (m, 4H, H-2, 6), 1.26 (d, *J*=6.0 Hz, 6H, H-9a, 9b); ^{13}C NMR (75 MHz, acetone-d_6) δ: 174.6, 167.1, 166.8, 149.0, 148.9, 146.4 (2C), 146.2, 146.0, 127.6, 127.5, 122.7, 122.6, 116.4 (2C), 115.8, 115.5, 115.2, 115.1, 75.0, 74.5, 69.4, 69.3, 65.8, 41.2, 36.7, 21.9 (2C); ESI-MS (*m/z*): 581.5[M+Na]$^+$, 1139.3[2M+Na]$^+$; HRMS-ESI (*m/z*): calcd for $C_{28}H_{31}O_{12}$ 559.1810, found 559.1806[M+H]$^+$.

Synthesis of *n*-butyl 3,5-di-*O*-caffeoylquinate (LS-5): Followed the process as described above. Isochlorogenic acid B (0.1 g, 0.19 mmol), thionylchloride (0.03 ml, 0.38 mmol), 5 mL of dried *n*-butyl alcohol were added. The final purification by RP-HPLC (acetonitrile:H_2O=2:3, *V:V*) to give LS-5 as black powder (77.9 mg, 70.3%). ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.57 (d, *J*=12.0 Hz, 1H, H-7'), 7.52 (d, *J*=12.0 Hz, 1H, H-7''), 7.15 (m, 2H, H-2', 2''), 6.99 (m, 2H, H-6', 6''), 6.85 (m, 2H, H-5', 5''), 6.30 (d, *J*=12.0 Hz, 1H, H-8'), 6.25 (d, *J*=12.0 Hz, 1H, H-8''), 5.60 (m, 1H, H-3), 5.07 (dd, *J*=6.3, 2.4 Hz, 1H, H-4), 4.39 (m, 1H, H-5), 4.13 (t, *J*=6.0 Hz, 2H, H-8), 2.07-2.38 (m, 4H, H-2, 6), 1.65 (quint, *J*=6.0 Hz, 2H, H-9), 1.42 (sext, *J*=6.0 Hz, 2H, H-10), 0.92 (t, *J*=6.0 Hz, 3H, H-11); ^{13}C NMR(75 MHz, acetone-d_6) δ: 175.1, 167.0, 166.8, 148.8, 148.7, 146.3 (2C), 146.1, 146.0, 127.7, 127.6, 122.7, 122.6, 116.4 (2C), 115.9, 115.6, 115.2 (2C), 75.0, 74.6, 69.4, 65.9, 65.6, 41.3, 36.7, 31.4, 19.8, 14.1; ESI-MS (*m/z*): 571.7[M-H]$^-$.

Synthesis of iso-butyl 3,5-di-*O*-caffeoylquinate (LS-6): Followed the process as described above. Isochlorogenic acid B (0.1 g, 0.19 mmol), thionylchloride (0.03 ml, 0.38 mmol), 5 mL of dried *p*-butyl alcohol were added. The final purification by RP-HPLC (acetonitrile:H_2O=2:3, *V:V*) gave LS-6 as brown oil (75.2 mg, 67.8%). $[\alpha]_D^{25}$ -39.8°(c=1.0, MeOH); ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.57 (d, *J*=12.0 Hz, 1H, H-7'), 7.52 (d, *J*=12.0 Hz, 1H, H-7''), 7.15 (m, 2H, H-2', 2''), 6.99 (m, 2H, H-6', 6''), 6.85 (m, 2H, H-5', 5''), 6.30 (d, *J*=12.0 Hz, 1H, H-8'), 6.25 (d, *J*=12.0 Hz, 1H, H-8''), 5.60 (m, 1H, H-3), 5.07 (dd, *J*=6.3, 2.4 Hz, 1H, H-4), 4.39 (m, 1H, H-5), 3.92 (d, *J*=6.0 Hz, 2H, H-8), 2.07-2.38 (m, 4H, H-2, 6), 1.98 (m, 1H, H-9), 0.96 (d, *J*=6.0 Hz, 6H, H-10a, 10b); ^{13}C NMR (75 MHz, acetone-d_6) δ: 175.1, 167.0, 166.8, 148.8, 148.7, 146.3 (2C), 146.1, 146.0, 127.7, 127.6, 122.7, 122.6, 116.4 (2C), 115.9, 115.6, 115.2 (2C), 75.1, 74.7, 71.8, 69.4, 65.8, 41.4, 36.8, 28.6, 19.4 (2C); ESI-MS (*m/z*): 595.7[M+Na]$^+$, 1167.4[2M+Na]$^+$; HRMS-ESI (*m/z*): calcd for $C_{29}H_{33}O_{12}$ 573.1967, found 573.1978[M+H]$^+$.

Synthesis of benzyl 3,5-di-*O*-caffeoylquinate (LS-7): Followed the process as described above. Isochlorogenic acid B (0.1 g, 0.19 mmol), thionylchloride (0.03 ml, 0.38 mmol), 5 mL of dried benzylalcohol were added. The final purification by RP-HPLC (acetonitrile:H_2O=2:3, *V:V*) gave LS-7 as yellow oil (83.9 mg, 71.5%). $[\alpha]_D^{25}$ -29.0°(c=1.0, MeOH); ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.57 (d, *J*=12.0 Hz, 1H, H-7'), 7.52 (d, *J*=12.0 Hz, 1H, H-7''), 7.32-7.46 (m, 5H, H-10a, 10b, 11a, 11b, 12), 7.15 (m, 2H, H-2', 2''), 6.99 (m, 2H, H-6', 6''), 6.84 (m, 2H, H-5', 5''), 6.30 (d, *J*=12.0 Hz, 1H, H-8'), 6.24 (d, *J*=12.0 Hz, 1H, H-8''), 5.61 (m, 1H, H-3), 5.19 (s, 2H, H-8), 5.09 (dd, *J*=6.3, 2.4 Hz, 1H, H-4), 4.39 (m, 1H, H-5), 2.13-2.44 (m, 4H, H-2, 6); ^{13}C NMR (75 MHz, acetone-d_6) δ: 174.8, 167.0, 166.8, 148.9, 148.8, 146.4 (2C), 146.2, 146.0, 137.3, 129.3 (2C), 128.9, 128.8 (2C), 127.6, 127.5, 122.7, 122.6, 116.4 (2C), 115.8, 115.5, 115.2 (2C), 74.9, 74.7, 69.3, 67.3, 65.9, 41.1, 36.7; ESI-MS (*m/z*): 605.8[M-H]$^-$; HRMS-ESI (*m/z*): calcd for $C_{32}H_{29}O_{12}$ 605.1664, found 605.1666 [M-H]$^-$.

Synthesis of *n*-amyl 3,5-di-*O*-caffeoylquinate (LS-8): Followed the process as described above. Isochlorogenic acid B (0.1 g, 0.19 mmol), thionylchloride (0.03 ml, 0.38 mmol), 5 mL of dried *n*-pentanol were added. The final purification by RP-HPLC (acetonitrile:H_2O=9:11, *V:V*) gave LS-8 as brown oil (78.9 mg, 69.5%). $[\alpha]_D^{25}$ -17.7°(c=1.0, MeOH); ^{1}H NMR (300 MHz, CD_3OD) δ: 7.58 (d, *J*=12.0 Hz, 1H, H-7'), 7.53 (d, *J*=12.0 Hz, 1H, H-7''), 7.03 (m, 2H, H-2', 2''), 6.90 (m, 2H, H-6', 6''), 6.75 (m, 2H, H-5', 5''), 6.30 (d, *J*=12.0 Hz, 1H, H-8'), 6.24 (d, *J*=12.0 Hz, 1H, H-8''), 5.63 (m, 1H, H-3), 5.03 (dd, *J*=6.3, 2.4 Hz, 1H, H-4), 4.33 (m, 1H, H-5), 4.16 (t, *J*=6.0 Hz, 2H, H-8), 2.09-2.38 (m, 4H, H-2, 6), 1.70 (quint, *J*=6.0 Hz, 2H, H-9), 1.39 (m, 4H, H-10, 11), 0.94 (t, *J*=6.0 Hz, 3H, H-12); ^{13}C NMR (75 MHz, CD_3OD) δ: 175.9, 168.7, 168.6, 149.8 (2C), 147.5 (2C), 147.0 (2C), 127.9, 127.8, 123.4, 123.3, 116.6 (2C), 115.3, 115.2, 115.1, 115.0, 75.9, 75.4, 70.0, 66.8, 66.2, 41.6, 37.0, 29.5, 29.3, 23.5, 14.5; ESI-MS (*m/z*): 609.6 [M+Na]$^+$, 1195.1[2M+Na]$^+$; HRMS-ESI (*m/z*): calcd for $NaC_{30}H_{34}O_{12}$ 609.1942, found 609.1968[M+Na]$^+$.

Synthesis of *n*-hexyl 3,5-di-*O*-caffeoylquinate (LS-9): Followed the process as described above. Isochlorogenic acid B (0.1 g, 0.19 mmol), thionylchloride (0.03 ml, 0.38 mmol), 5 mL of dried *n*-hexanol were added. The final purification by RP-HPLC (acetonitrile:H_2O=9:11, *V:V*) gave LS-9 as slight green oil (79.9 mg, 68.7%). $[\alpha]_D^{25}$ -21.0°(c=1.0, MeOH); ^{1}H NMR (300 MHz, acetone-d_6) δ: 7.57 (d, *J*=12.0 Hz, 1H, H-7'), 7.52 (d, *J*=12.0 Hz, 1H, H-7''), 7.15 (m, 2H, H-2', 2''), 6.99 (m, 2H, H-6', 6''), 6.85 (m, 2H, H-5', 5''), 6.29 (d, *J*=12.0 Hz, 1H, H-8'), 6.24 (d, *J*=12.0 Hz, 1H, H-8''), 5.59 (m, 1H, H-3), 5.07 (dd, *J*=6.3, 2.4 Hz, 1H, H-4), 4.38 (m, 1H, H-5), 4.13 (t, *J*=6.0 Hz, 2H, H-8), 2.09-2.38 (m, 4H, H-2, 6), 1.67 (quint, *J*=6.0 Hz, 2H, H-9), 1.29-1.42 (m, 6H, H-10, 11, 12), 0.89 (t, *J*=6.0 Hz, 3H, H-13); ^{13}C NMR (75 MHz, acetone-d_6) δ: 175.1, 167.0, 166.8, 148.9 (2C), 146.4, 146.1, 146.0 (2C),

127.6, 127.5, 122.7, 122.6, 116.5 (2C), 115.9, 115.6, 115.2 (2C), 75.0, 74.7, 69.4, 65.9, 65.8, 41.3, 36.8, 32.2, 29.3, 26.3, 23.3, 14.4; ESI-MS (*m/z*): 623.5[M+Na]$^+$, 1223.1[2M+Na]$^+$; HRMS-ESI (*m/z*): calcd for $C_{31}H_{37}O_{12}$ 601.2280, found: 601.2286[M+H]$^+$.

Biological process

Viruses and cells: Respiratory syncytial virus (RSV) (Long strain, ATCC-VR-26) was purchased from Medicinal Virology Institute, Wuhan University, China. The virus was cultured in human larynx epidermoid carcinoma cell line (HEp-2 cells) with titer of approximately 5×10^6 plaque-forming unit (pfu)/ml. Cultures were aliquoted and kept frozen at -70°C until use.

HEp-2 cells were grown in Eagle's minimum essential medium (MEM) (Gibco) supplemented with 10% fetal bovine serum (FBS) (Gibco), 25 μg/ml gentamicin (Sigma), and 200 mM L-glutamine (Sigma), which is the growth medium (10%GM). RSV-infected cells (HEp-2 cells) were maintained in MEM with 1% FBS, 25 μg/ml gentamicin, and 200 mM L-glutamine, which is the maintenance medium (1%MM). All the cells were cultured at 37°C in a humidified atmosphere supplied with 5% CO_2.

Plaque reduction assay: The inhibitory effects of 3,4-DCQA and its derivatives (LS-1 to LS-9) on the replication of RSV in HEp-2 cells were tested by the plaque reduction assay, which was performed according to the method described previously with some modifications [30]. Briefly, monolayer of HEp-2 cells was grown on 12-well plate. Approximate 80 pfu of RSV was added to the cells, immediately followed by the addition of various concentrations of the sample. The plate was incubated in 5% CO_2 at 37°C for 2 h with intermittent rocking at 15-min intervals, and then overlaid with agarose overlay medium containing various concentrations of the test sample. After incubation for 5 days at 37°C, the infected cells were fixed with 10% formalin, stained with 1% crystal violet, and the number of plaques was counted. The wells overlaid with agarose medium without test sample were used as the control. The percentage of inhibition of plaque formation was calculated as follows: [(mean number of plaques in control)−(mean number of plaques in sample)] × 100/(mean number of plaques in control). The values of IC_{50}, which was the concentration of test sample required to inhibit up to 50% of virus growth as compared with the virus control group, were estimated from the graphic plots of the data. Ribavirin (Sigma, St. Louis) was used as a positive control drug in the anti-RSV study.

MTT reduction assay: Cellular viability was assayed with MTT method [31,32]. In the assay, different concentrations of samples (100 μl) were applied to the wells of 96-well plate containing confluent cell monolayer in triplicate, while the dilution medium without the sample was used as the control. After 3 days of incubation, 12 μl of the MTT (3-[4,5-dimethyl-thiazol-2-yl]-2,5-diphenyl tetrazolium bromide) solution (5 mg/ml in phosphate buffered saline) was added to each well. The plate was further incubated for 3 h to allow the formation of MTT formazan. After removing the medium, 100 μl of dimethyl sulfoxide (DMSO) was added to dissolve the formazan crystals. After 15 min, the content in the wells was homogenized on a microplate shake. The optical densities (OD) were then read at a microplate spectrophotometer at double wavelengths of 540 and 690 nm, respectively. The median cytotoxic concentration (CC_{50}) was calculated as the concentration of the sample that decreased the number of viable cells to 50% of the cell control through the OD values of viable cells in comparison with non-viable cells.

Results and Discussion

Chemistry

The syntheses of alkyl 3,4-di-*O*-caffeoylquinates were outlined in Scheme 1 using isochlorogenic acid B as starting material. Normally, in order to esterify 1-carboxylic group selectively, all the hydroxyl groups, including 1-OH, 2-OH, and all phenolic groups should be protected. However, a strategy called "saturated attack" was applied, in which large excess of alcohol was used as reagent as well as solvent. Firstly, *N,N'*-dicyclohexylcarbodiimide (DCC) was tried as coupling reagent. With the application of DCC, methyl 3,4-di-*O*-caffeoylquinate (LS-1) was successfully prepared with a yield of 55.3%. Unfortunately, other 3,4-di-*O*-caffeoylquinate derivatives even including LS-2 could not be synthesized by using DCC.

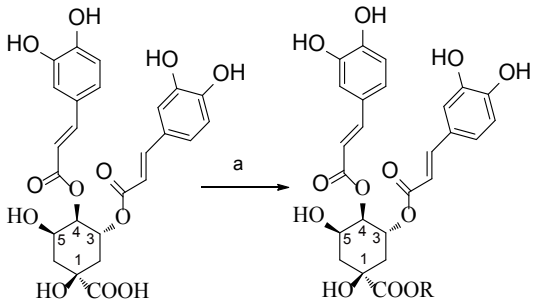
Later, we found out an effective coupling reagent, namely thionylchloride ($SOCl_2$), that could be used for the preparation of all alkyl 3,4-di-*O*-caffeoylquinates including LS-1 to LS-9 with yields of 56%-72%. This is a clean reaction. Under 0°C, 3,4-DCQA was turned into active 3,4-di-*O*-caffeoylquinyl chloride by $SOCl_2$, and then was attacked saturatedly by alcohol with the release of HCl and SO_2. Excess alcohol and $SOCl_2$ were removed easily by evaporation at room temperature.

The reaction yields of LS-2 to LS-9 were almost equal, which were around 70%. However, synthesis of compound LS-1 showed unexpectedly low yield. We suggested that part of methyl 3,4-di-*O*-caffeoylquinate (LS-1) may probably be dimerized to form a lactide (Figure 3), which was confirm by mass spectrometry. Other 3,4-di-*O*-caffeoylquinates showed less active to form lactide because of steric hindrance.

Anti-RSV activities of alkyl 3,4-di-*O*-caffeoylquinates

By applying plaque reduction assay, the anti-RSV activities of all the compounds were evaluated. As shown in Table 1, esterification of 1-carboxylic group in 3,4-DCQA increased its anti-RSV activity. The IC_{50} values of the derivatives were 2.9 to 7.8 times less than that of 3,4-DCQA itself. LS-4, namely the isopropyl 3,4-di-*O*-caffeoylquinate, was confirmed as the most active compound with IC_{50} value of 0.3 μM. It was indicated that hydrophobic groups with 2 to 4 carbon chain length were optimal for the enhancement of anti-RSV activity (LS-2 to LS-7).

As pointed out by Li et al. [24], 3,4-DCQA exhibited less cytotoxicity against HEp-2 cells with mean (50%) cytotoxic concentration (CC_{50}



Compounds	R	Yield (%)
LS-1	Me	56.7
LS-2	Et	68.7
LS-3	*n*-Pr	67.5
LS-4	*i*-Pr	66.3
LS-5	*n*-Bu	70.3
LS-6	*i*-Bu	67.8
LS-7	Bn	71.5
LS-8	*n*-Amyl	69.5
LS-9	*n*-hex	68.7

Scheme 1: Synthesis of alkyl 3,4-Di-*O*-caffeoyl quinates. Conditions and reagents: ROH (excess), 2 eq $SOCl_2$, -15°C→r.t., overnight. Yields: 56%-72%.

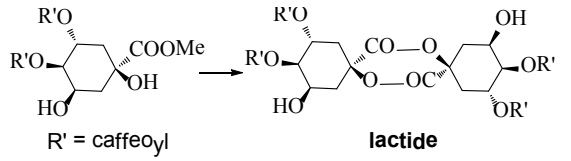


Figure 3: Methyl 3,4-Di-*O*-caffeoyl quinate may dimerize to lactide.

Compounds	Anti-RSV activity[a] IC_{50} (μM)[b]	Cytotoxicity[c] CC_{50} (μM)[d]	Selective index[e] (SI)
3,4-DCQA	2.33	1160	497.8
LS-1	0.7	>500	>714.3
LS-2	0.5	150	300.0
LS-3	0.37	85	229.7
LS-4	0.3	85	283.3
LS-5	0.5	5	10.0
LS-6	0.4	2.5	6.25
LS-7	0.5	40	80.0
LS-8	0.8	50	62.5
LS-9	0.8	25	31.2
Ribavirin	3.1	50	16.1

[a]The data of anti-RSV activity were measured by plaque reduction assay; [b]IC_{50}: Mean (50%) inhibitory concentration; [c]The data of cytotoxicities were tested by MTT method; [d]CC_{50}: Mean (50%) cytotoxic concentration; [e]SI values were calculated from CC_{50}/IC_{50}

Table 1: Anti-RSV activities and cytotoxicities of alkyl 3, 4-di-*O*-Caffeoylquinates.

value) of 1160 μM. Therefore, CC_{50} values of all the compounds were also tested. As a result, LS-1 to LS-9 showed greater cytotoxicity than 3,4-DCQA, and LS-6 was identified as the most toxic compound with CC_{50} value of 2.5 μM. Basically, it was indicated that esterification of 3,4-DCQA with longer carbon chain alcohol results in greater cytotoxicity.

So far, considering both factors of anti-RSV activity and cytotoxicity, LS-4 may be regarded as the optimal lead compound with selective index (SI) of 283.3. It is far better than ribavirin, which the SI value was 16.1 only.

Explanation of R effect on anti-RSV activity from molecular docking study

Based on molecular docking study, ribavirin did not bind to RSV-F well (Figure 4). The total score was only 5.1869 (Table 2). This is in agreement with the fact that ribavirin targets on cellular inosine monophosphate dehydrogenase, which leads to the inhibition of virus replication [33]. On the contrary, 3,4-DCQA bound to RSV-F well with a total score of 7.5941. As shown in Figure 4, there were three hydrogen bonds between 3,4-DCQA and RSV-F, which were 3'-OH/Val150, 4'-OH/Ala168, and 1-OH/Lys103, respectively. In accordance with the anti-RSV activity results (Table 1), the total scores of all the esterified derivatives, except LS-3, were greater than that of 3,4-DCQA, indicating stronger binding affinities to RSV-F. Although the total score of LS-3 was a little bit less than that of 3,4-DCQA (6.8001 to 7.5941), its polarity was lower (5.2635 to 6.182). This low polarity benefited the binding of LS-3 to RSV-F.

LS-7 possessed the highest score (12.4499), and LS-5 the second. However, their molecular polarities were quite high, which were 7.5841 and 9.5321, respectively. This might be the reason why they were not the most active inhibitor. It was suggested that appropriate molecular polarity will be helpful for the binding of inhibitor with RSV fusion protein.

Stability

As mentioned above, LS-4 was identified as the optimal anti-RSV candidate among all the prepared derivatives. Therefore, the stability of LS-4 was investigated using RP-HPLC method. The standard concentration curve was made firstly. It was found that the peak areas are linearly correlated with concentrations quite well with correlation coefficience square (R^2) value of 0.9999 at the range of 0.015-1.0 mg/ml (Figure 5A). Then, stability of LS-4 in the solvents of pure water, artificial gastric juice (AGJ), and artificial intestinal juice (AIJ) was evaluated, respectively. Quite interestingly, LS-4 showed the most stable in the environment of AGJ with hydrolysis rate of 24.9% after 6-hour incubation at 37°C (Figure 5B), while in pure water, the rate was 54.6%, and in AIJ, the rate was 36.7%.

The hydrolysis speed of LS-4 in water was increased very fast after the first 2-hour incubation. We suggested that the weak acidic proton produced from phenolic groups (caffeoyl) might self-catalyze the hydrolysis process. However, strong acid condition (pH 1.0 in AGJ) may inhibit this process. This hydrolysis inhibition may probably be ascribed to two effects. One is the dissociation inhibition against phenolic groups. Another is the inactivation of water, which become less nucleophilic.

Conclusions

In summary, nine alkyl 3,4-Di-*O*-caffeoyl quinates have been synthesized. By applying plaque reduction assay, the anti-RSV activities of all the compounds were evaluated. The IC_{50} values of the derivatives were 2.9 to 7.8 times less than that of 3,4-DCQA itself. And isopropyl 3,4-Di-*O*-caffeoyl quinate was confirmed as the most active compound with IC_{50} value of 0.3 μM. It was indicated that hydrophobic groups with 2 to 4 carbon chain length were optimal for the enhancement of anti-RSV activity. However, incorporation of R group increases the cytotoxicity, all the derivatives showed greater cytotoxicity than 3,4-DCQA itself. Except LS-5, LS-6 and LS-9, all the derivatives have less toxicity than

Compounds	Total_score	Crash	Polar
3,4-DCQA	7.5941	-2.0966	6.182
LS-1	7.5354	-2.8502	7.4607
LS-2	7.7698	-2.3217	3.005
LS-3	6.8001	-2.3472	5.2635
LS-4	7.6384	-1.6662	5.575
LS-5	12.0591	-2.5909	9.5321
LS-6	8.2606	-1.5548	5.1911
LS-7	12.4499	-1.91	7.5841
LS-8	9.3398	-1.7944	3.432
LS-9	8.635	-2.1796	6.1272
Ribavirin	5.1869	-0.6963	5.0839

Table 2: Docking parameters of 3,4-DCQA derivatives to RSV fusion protein.

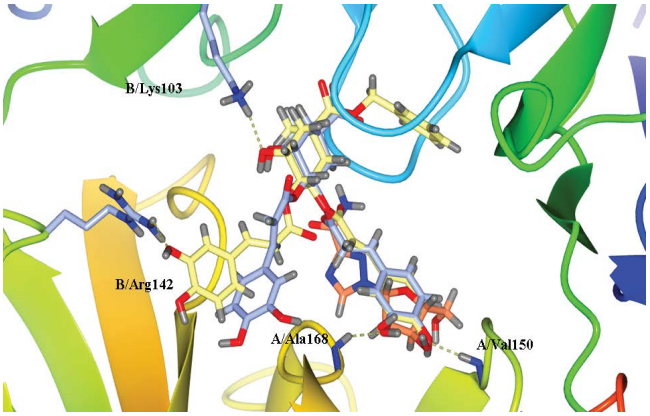


Figure 4: Comparison of the interactions of **LS-7** (lemon), **ribavirin** (coral), and **3,4-DCQA** (ice blue) with RSV fusion protein (PDB: 3IXT). Residues which could form hydrogen bond with the substrates were labelled.

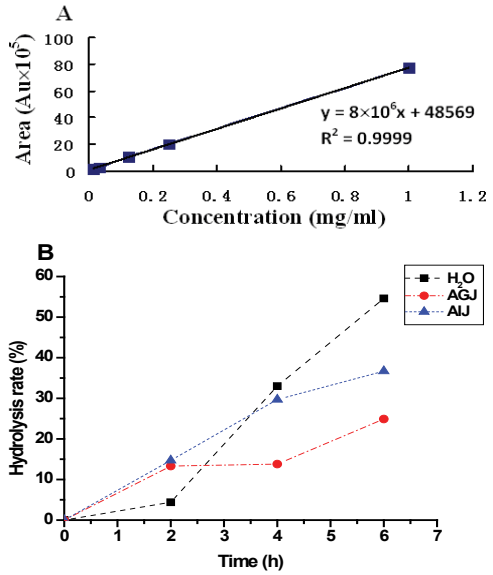


Figure 5: Stabilities of **LS-4** in pure water, artificial gastric juice (AGJ), and artificial intestinal juice (AIJ). (**A**) Standard concentration curve of **LS-4**. Peak areas corresponding with exact concentrations of 1.0, 0.25, 0.125, 0.03125, and 0.015063 mg/ml were plotted against concentrations. Reversed-phase high-performance liquid chromatography (RP-HPLC) was performed on a Cosmosil column (C_{18}, 5 Å, 4.6 × 250 mm) using an LC-100 fluid unit (Shanghai Wufeng Inc., China) with a tunable LC-100 UV detector. (**B**) Stabilities of LS-4 in pure water, artificial gastric juice (AGJ), and artificial intestinal juice (AIJ) at 37°C. Analyses of samples were scheduled at 0, 2.0, 4.0, and 6.0 h respectively, using RP-HPLC method.

ribavirin. Furthermore, stabilities of LS-4 in pure water, AGJ, and AIJ was evaluated, respectively. It was shown that LS-4 is stable in AGJ. The hydrolysis rate is 24.9% after 6 hours incubation at 37°C.

Acknowledgments

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