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Chemical Modification of Rupestonic Acid and Preliminarily *In Vitro* Antiviral Activity Against Influenza A_3 and B Viruses

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To improve the biological activities of rupestonic acid, 21 new rupestonic acid fatty ester derivatives (**2a-2h**) and aromatic ester derivatives (**2i-2u**) were synthesized and preliminarily evaluated for their anti-influenza activity *in vitro* by the national center for drug screening of China, using the Oseltamivir and Ribavirin as reference drugs. The results showed that **2l** (IC_{50} = 0.5 μmol/L) exhibited potent anti-influenza A_3 viral activity among the synthesized compounds and was 10-fold more potent than that of the reference drug Oseltamivir (IC_{50} = 5.1 μmol/L).

Key Words : Synthesis, Rupestonic acid ester derivatives, Anti-influenza virus

Introduction

Influenza is an acute infectious disease caused by a member of the orthomyxovirus family: influenza virus A, B or to a much lesser extent, influenza virus C. Epidemic influenza continues to be associated with significant morbidity in the general population, and mortality in the elderly and other high risk patients. Although the case fatality rate averages less than 0.01%, tens of thousands of deaths occur each year. Control through immunization programs has not been possible due to incomplete protective efficacy and antigenic variations that occur frequently.

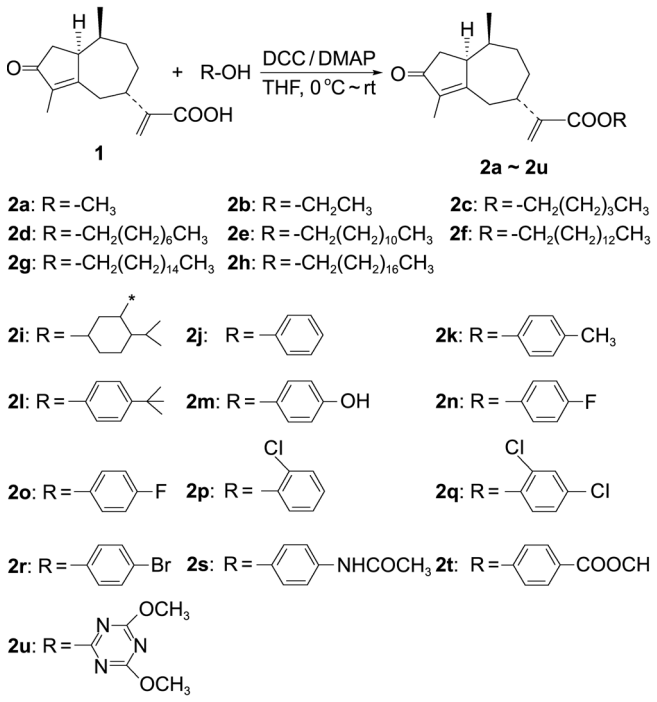
In recent years, the persistence of the strain of influenza H5N1 (avian influenza) in many Asian countries and its ability to cause fatal infections in humans has raised serious concerns. And the novel strain of influenza virus H1N1 (swine flu) arouse in 2009 and persistently spread globally and caused fatal death. Oseltamivir has been orally used for protection against and treatment of influenza, and regarded as the effective drug in clinical. But it is resistant to the strain of influenza H5N1 (avian influenza).[1] Thus it is an urgent demand to find new inhibitors, especially to find the novel and more active inhibitors from the natural products.

Artemisia rupestris L. (Chinese name is Yizhihao) is one of Chinese traditional herbal medicines long been used in folk of Xinjiang of China. It is known to be effective as antiallergic,[2] anti-tumour,[3] anti-inflammatory, anti-bacterial, antidote agents.[4,5] (5*R*,8*R*)-2-(3,8-dimethyl-2-oxo-1,2,4,5,6,7,8,8α-octahydroazulen-5-yl)acrylic acid[6] (named rupestonic acid, compound **1**) is a sesquiterpene with multifunctional groups, isolated from *Artemisia rupestris* L. Sesquiterpenes always exhibit considerable biological activities. Sheu Jyh-Horng and his coworkers have reported that some sesquiterpeneses exhibit potent cytotoxicity toward P-388, A549 and HT-26 cancer cell lines.[7] Amy E. Wright and her coworkers have also reported that the sesquiterpenes hydroquinone and its acetate derivative exhibited higher activities against the P-388 tumor cell line and influenza strain PR-8.[8] Thus we have tested the rupestonic acid against influenza A_3/jifang/90/15 and B/jifang/97/13 viruses. The results showed that it exhibits higher activity against influenza viral B (TC_{50} = 1044.4 μM, IC_{50} = 115.7 μM, SI = 9). Thus we modified its structure to obtain the more biological significance.

Based on our previous study,[9-11] in this work, we mainly modified the carboxyl group of the rupestonic acid and firstly synthesized 21 rupestonic acid derivatives **2a-2u** in the presence of DCC/DMAP. These new compounds were confirmed by the IR, ^{1}H NMR and ESI-MS spectra data, and then assayed their *in vitro* activities against influenza A_3, B viruses comparing the positive drugs Ribavirin (RBV) and Oseltamivir.

Experimental Section

Chemistry. All melting points were determined on Yanaco MP-300 micro melting points apparatus and values are uncorrected; ^{1}H NMR spectra datas were recorded on a varian inova-400 spectrometer, using the tetramethylsilane (TMS) as an internal reference and $CDCl_3$ as the solvent; ESI-MS were performed on a HP1100 LC/MS; Rupestonic acid was isolated from the *Artemisia rupestris* L (purity:over 98%); Dicyclohexyl Carbodiimide (DCC), 1-Hydroxybenzotriazole (HOBt), 4-dimethylaminopyridine (DMAP) were purchased from Shanghai reagent Company, China; the positive drug ribavirin(RBV)(obtained from Zhejiang Kangyu drug Co. Ltd., China) and Oseltamivir (obtained from shanghai drug Co. Ltd., China); A_3/Jifang/90/15 and B/Jifang/97/13 viruses (obtained from Beijing viral institute); Other chemicals are commercially available and used without further purification. THF was distilled from sodium and benzophenone before used.



Scheme 1

General Synthetic Procedure for the Preparation of Rupestonic Acid Derivatives (2a-2u, Scheme 1)[12]**.** Compound **1** (0.124 g, 0.5 mmol), DCC (0.11 g, 0.55 mmol) and (0.08 g, 0.6 mmol) HOBt were added into a 25 mL one necked round bottom flask with 5 mL dry THF. The mixture was stirred under cold bath for about 10 min, DMAP (0.07 g, 0.55 mmol) was added into the reaction system. The mixture was stirred under the cold bath for 30 min, subsequently, 0.6 mmol R-OH was added. The mixture was stirred for 30 min under cold bath then temperature rose naturally to room temperature. The completion of reaction was judged from the simple TLC analysis. The mixture was evaporated under reduced pressure and the residual purified directly by column chromatography (EtOAc/Petroleum ether: 5:1 → 2:1) to give the desired compounds **2a-2u**.

Compound 2a: Light yellow oil, yield: 75%. IR(KBr) ν 3032, 2951, 2924, 1712, 1662, 1566, 1450, 1375, 1339, 1240, 1145 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.66 (d, J = 7.3 Hz, 3H, CH_3), 1.49 (s, 3H, CH_3), 1.60-1.69 (m, 4H, $2CH_2$), 1.97-2.08 (m, 2H), 2.15-2.31 (m, 1H), 2.33-2.45 (m, 1H), 2.50-2.58 (m, 2H), 3.21 (s, 1H), 3.77 (s, 3H, CH_3), 5.63 (s, 1H), 6.22 (s, 1H); MS (*m/z*, 100%) 262 ($[M]^+$, 100), 263 ($[M+1]^+$, 20), 285 ($[M+23]^+$, 10).

Compound 2b: Yellow oil, yield: 87.6%. IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1566, 1450, 1375, 1339, 1240, 1145 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.66 (d, J = 7.3 Hz, 3H, CH_3), 1.29 (s, 3H, CH_3), 1.31(t, J = 8.5 Hz, 3H,CH_3), 1.60-1.69 (m, 4H, $2CH_2$), 1.97-2.08 (m, 2H), 2.15-2.31 (m, 1H), 2.33-2.45 (m, 1H), 2.50-2.58 (m, 2H), 3.21 (brs, 1H), (q, J = 6.5, 7.8, 8.6 Hz, 2H, CH_2), 5.57 (s, 1H), 6.22 (s, 1H); MS (*m/z*, 100%) 277 ($[M+1]^+$, 96), 299 ($[M+23]^+$, 100), 315 ($[M+39]^+$, 10).

Compound 2c: Colorless oil, yield: 68.6%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662,1618, 1565, 1450, 1375, 1339, 1240, 1145 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.66 (d, J = 7.3 Hz, 3H, CH_3), 0.88 (t, J = 8.2 Hz, 3H, CH_3), 1.41 (m, 4H), 1.62 (s, 3H), 1.67-1.71 (m, 4H), 1.73 (m, 1H), 1.78-1.83 (m, 2H), 2.05 (d, J = 18.2 Hz, 2H), 2.14 (m, 1H), 2.42-2.49 (m, 1H), 2.57-2.62 (m, 1H), 2.82-2.95 (m, 2H), 3.20 (m, 1H), 5.61 (s, 1H), 6.22 (s, 1H); MS (*m/z*, 100%) 318 ($[M]^+$, 100), 319 ($[M+23]^+$, 25).

Compound 2d: Light yellow oil, yield: 75.6%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.66 (d, J = 7.3 Hz, 3H, CH_3), 0.85 (t, J = 9.2 Hz, 3H, CH_3), 1.25-1.39 (m, 8H), 1.65 (s, 3H, CH_3), 1.69 (m, 4H), 1.78-1.86 (m, 4H), 2.04 (d, J = 10.2 Hz, 1H), 2.14 (m, 1H), 2.43-2.46 (m, 1H), 2.51 (m, 1H), 2.83-2.95 (m, 2H), 3.19 (m, 1H), 3.54 (m, 2H), 5.62 (s, 1H), 6.23 (s, 1H); MS (*m/z*, 100%): 361 ($[M+1]^+$, 100), 383 ($[M+23]^+$, 30).

Compound 2e: Colorless oil, yield: 61.1%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145, 918 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.66 (d, J = 7.3 Hz, 3H, CH_3), 0.79-0.85 (m, 3H, CH_3), 1.22-1.41 (m, 14H, $7CH_2$), 1.51-1.58 (m, 4H), 1.66 (s, 3H, CH_3), 1.75-1.85 (m, 2H), 2.02-2.18 (m, 2H), 2.42-2.51 (m, 1H), 2.58-2.65 (m, 1H), 2.82-2.95 (m, 2H), 3.41 (m, 1H), 3.61-3.64 (m, 4H), 4.15-4.18 (m, 2H), 5.62 (s, 1H), 6.22 (s, 1H); MS (*m/z*, 100%) 417 ($[M+1]^+$, 100), 439 $(M+23)^+$ (25).

Compound 2f: White solid, mp 28-30 °C, yield: 51.4%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.66 (d, J = 7.3 Hz, 3H, CH_3), 0.77-0.86 (m, 3H, CH_3), 1.23-1.42 (m, 18H, $9CH_2$), 1.51-1.58 (m, 4H), 1.66 (s, 3H, CH_3), 1.75-1.85 (m, 2H), 2.02-2.18 (m, 2H), 2.42-2.51 (m, 1H), 2.58-2.65 (m, 1H), 2.82-2.95 (m, 2H), 3.42 (m, 1H), 3.61-3.64 (m, 4H), 4.15-4.18 (m, 2H), 5.62 (s, 1H), 6.22 (s, 1H); MS (*m/z*, 100%): 445 ($[M+1]^+$, 100), 467 ($[M+23]^+$, 20).

Compound 2g: White solid, mp 49-50 °C, yield: 73.6%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.65 (d, J = 7.3 Hz, 3H, CH_3), 0.78-0.88 (m, 3H, CH_3), 1.25-1.42 (m, 22H, $11CH_2$), 1.51-1.58 (m, 4H), 1.66 (s, 3H, CH_3), 1.75-1.85 (m, 2H), 2.02-2.18 (m, 2H), 2.42-2.51 (m, 1H), 2.58-2.65 (m, 1H), 2.82-2.95 (m, 2H), 3.42 (m, 1H), 3.61-3.64 (m, 4H), 4.15-4.18 (m, 2H), 5.62 (s, 1H), 6.22 (s, 1H); MS (*m/z*, 100%) 473 ($[M+1]^+$, 100), 495 ($[M+23]^+$, 15).

Compound 2h: White solid, mp 51-52 °C, yield: 68.8%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145, 918 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.64 (d, J = 7.3 Hz, 3H, CH_3), 0.68-0.89 (m, 3H, CH_3), 1.11-1.30 (brs, 26H, $13CH_2$), 1.52-1.56 (m, 4H), 1.68 (s, 3H, CH_3), 1.75-1.85 (m, 2H), 2.02-2.18 (m, 2H), 2.42-2.51 (m, 1H), 2.58-2.65 (m, 1H), 2.82-2.95 (m, 2H), 3.42 (m, 1H), 3.61-3.64 (m, 4H), 4.15-4.18 (m, 2H), 5.62 (s, 1H), 6.22 (s, 1H); MS (*m/z*, 100%) 501 ($[M+1]^+$, 70). 523 ($[M+23]^+$, 10).

Compound 2i: Light yellow oil, yield: 67.9%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.66

(d, J = 7.3 Hz, 3H, CH_3), 0.76-0.78 (m, 4H, $2CH_2$), 0.80 (s, 3H, CH_3), 0.82 (s, 3H, CH_3), 0.92 (s, 3H, CH_3), 1.66 (s, 3H, CH_3), 1.79-1.86 (m, 2H), 1.96-1.97 (m, 1H), 1.98-1.99 (m, 1H), 2.02-2.06 (m, 1H), 2.05-2.08 (m, 1H), 2.15-2.21 (m, 2H), 2.40-2.48 (m, 1H), 2.57-2.59 (m, 1H), 2.62-2.64 (m, 1H), 2.82-2.86 (m, 1H), 2.92-2.95 (m, 1H), 3.19-3.20 (m, 1H), 3.38-3.44 (m, 2H), 4.76 (m, 1H), 5.58 (s, 1H), 6.20 (s, 1H); MS (*m/z*, 100%) 387 ($[M+1]^+$, 100), 409 ($[M+23]^+$, 15).

Compound 2j: Colorless oil, yield: 59.4%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145, 918 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.66 (d, J = 7.3 Hz, 3H, CH_3), 1.63 (m, 1H), 1.68 (s, 3H, CH_3), 1.69-1.72 (m, 1H), 1.80-1.86 (m, 2H), 1.87-1.91 (m, 1H), 2.04-2.05 (m, 1H), 2.15-2.17 (m, 1H), 2.52-2.59 (m, 2H), 2.91-3.02 (m, 2H), 3.20-3.22 (m, 1H), 5.84 (s, 1H), 6.50 (s, 1H), 7.11-7.15 (m, 2H), 7.23-7.28 (m, 1H), 7.39-7.44 (m, 2H); MS (*m/z*, 100%) 324 ($[M]^+$, 100), 325 ($[M+1]^+$, 20), 347 ($[M+23]^+$, 18).

Compound 2k: White solid, mp 110-111 ºC, yield: 86.5%, ^1H NMR (400 MHz, $CDCl_3$) δ 0.67-0.69 (d, J = 8 Hz, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.72-1.89 (m, 4H), 2.03-2.05 (m, 1H), 2.14-2.16 (m, 1H), 2.38 (s, 3H, CH_3), 2.51-2.64 (m, 2H), 2.95-3.04 (m, 2H), 3.20-3.22 (m, 1H), 5.81 (s, 1H), 6.48 (s, 1H), 7.02 (d, J = 7.8 Hz, 2H), 7.20 (d, J = 8.6 Hz, 2H); MS (*m/z*, 100%) 339 ($[M+1]^+$, 60), 361 ($[M+23]^+$, 100).

Compound 2l: Colorless oil, yield: 77.9%, IR (KBr) υ 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145, 918 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.66 (d, J = 7.3 Hz, 3H, CH_3), 1.31 (s, 9H, $3CH_3$), 1.63 (s, 3H, CH_3), 1.65-1.81 (m, 4H), 2.04-2.08 (m, 1H), 2.14-2.16 (m, 1H), 2.53-2.64 (m, 2H), 2.91-3.04 (m, 2H), 3.21 (m, 1H), 5.82 (m, 1H), 6.48 (m, 1H), 7.03-7.07 (m, 2H), 7.39-7.43 (m, 2H); MS (*m/z*, 100%) 381 ($[M+1]^+$, 100), 403 ($[M+23]^+$, 80).

Compound 2m: White solid, mp 119-121 ºC, yield: 68.8%, ^1H NMR (400 MHz, $CDCl_3$) δ 0.64 (d, J = 7.3 Hz, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.75-1.88 (m, 4H), 2.08 (d, J = 16.0 Hz, 1H), 2.16 (m, 1H), 2.51-2.64 (m, 2H), 2.90-3.03 (m, 2H), 3.21 (m, 1H), 5.81 (s, 1H), 6.45 (s, 1H), 6.72 (s, 1H), 6.85 (d, J = 8.0 Hz, 2H), 6.98 (d, J = 8.0 Hz, 2H); MS (*m/z*, 100%) 339 ($[M-1]^-$, 45), 240 ($[M]^+$, 8).

Compound 2n: White solid, yield: 77.2%, mp 89-90 ºC; IR (KBr) ν 2959-2878, 1713, 1694, 1627, 1512, 1223, 1141, 826, 748 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.68 (d, J = 7.3 Hz, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.68-1.89 (s, 4H, $2CH_2$), 2.13-2.15 (m, 1H), 2.14 (m, 1H), 2.50-2.61 (m, 2H, CH_2), 2.91-2.94 (m, 1H), 2.99-3.01 (m, 1H), 3.18-3.19 (m, 1H), 5.86 (s, 1H), 6.49 (s, 1H), 7.06-7.10 (m, 2H), 7.35-7.39 (m, 2H); MS (*m/z*, 100%) 342 ($[M]^+$, 100).

Compound 2o: White solid, yield: 60.0%, mp 91-93 ºC, IR (KBr) ν 2959-2878, 1713, 1694, 1627, 1512, 1223, 1141, 826, 748 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.65 (d, J = 7.3 Hz, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.68-1.89 (brs, 4H, $2CH_2$), 2.13-2.15 (m, 1H), 2.14 (m, 1H), 2.50-2.61 (m, 2H, CH_2), 2.91-2.94 (m, 1H), 2.99-3.01 (m, 1H), 3.18-3.19 (m, 1H), 5.85 (s, 1H), 6.49 (s, 1H), 7.08 (d, J = 8.4 Hz, 2H), 7.10 (d, J = 8.6 Hz, 2H); MS (*m/z*, 100%) 358 ($[M]^+$, 78), 359 (100).

Compound 2p: Light yellow oil, yield: 51.6%, IR (KBr) ν 2959-2878, 1713, 1694, 1627, 1512, 1223, 1141, 826, 748 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.68 (d, J = 7.3 Hz, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.68-1.89 (s, 4H, $2CH_2$), 2.13-2.15 (m, 1H), 2.14 (m, 1H), 2.50-2.61 (m, 2H, CH_2), 2.91-2.94 (m, 1H), 2.99-3.01 (m, 1H), 3.18-3.19 (m, 1H), 5.88 (s, 1H), 6.56 (s, 1H), 7.19-7.24 (m, 2H), 7.30-7.34 (m, 1H), 7.46-7.48 (m, 1H); MS (*m/z*, 100%) 358 ($[M]^+$, 100).

Compound 2q: Light yellow oil, yield: 85.3%, IR (KBr) ν 2959-2878, 1713, 1694, 1627, 1512, 1223, 1141, 826, 748 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.68 (d, J = 7.3 Hz, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.68-1.89 (s, 4H, $2CH_2$), 2.13-2.15 (m, 1H), 2.14 (m, 1H), 2.50-2.61 (m, 2H, CH_2), 2.91-2.94 (m, 1H), 2.99-3.01 (m, 1H), 3.18-3.19 (m, 1H), 5.89 (s, 1H), 6.56 (s, 1H), 7.15 (d, J = 8.0 Hz, 2H), 7.28-7.31 (m, 1H), 7.48 (d, J = 8.6 Hz, 1H); MS (*m/z*, 100%) 392 ($[M]^+$, 100).

Compound 2r: Colorless oil, yield: 52.1%, IR (KBr) ν 2959-2878, 1713, 1694, 1627, 1512, 1223, 1141, 826, 748 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.69 (d, J = 7.3 Hz, 3H, CH_3), 1.67 (s, 3H, CH_3), 1.68-1.89 (s, 4H, $2CH_2$), 2.13-2.15 (m, 1H), 2.14 (m, 1H), 2.50-2.61 (m, 2H, CH_2), 2.91-2.94 (m, 1H), 2.99-3.01 (m, 1H), 3.18-3.19 (m, 1H), 5.86 (s, 1H), 6.49 (s, 1H), 7.01-7.05 (m, 2H), 7.50-7.54 (m, 2H); MS (*m/z*, 100%) 402 ($[M]^+$, 100).

Compound 2s: White solid, mp 168-170 ºC, yield: 91%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145, 918 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.67(d, J = 7.3 Hz, 3H, CH_3), 1.15-1.40 (m, 4H, $2CH_2$), 1.67 (s, 3H, CH_3), 1.81-1.83 (m, 1H), 2.04 (s, 1H), 2.18 (s, 3H, CH_3), 2.51-2.64 (m, 2H), 2.89-2.94 (m, 1H), 2.98-3.03 (m, 1H), 3.20-3.21 (m, 1H), 5.83 (s, 1H), 6.48 (s, 1H), 7.33 (s, 1H, NH), 7.10 (d, J = 8.0 Hz, 2H), 7.55 (d, J = 12.0 Hz, 2H); MS (*m/z*, 100%) 382 ($[M+1]^+$, 30), 404 ($[M+23]^+$, 100).

Compound 2t: Colorless oil, yield: 68.1%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145, 918 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.68 (d, J = 7.3 Hz, 3H, CH_3), 1.68 (s, 3H, CH_3), 1.72-1.75 (m, 1H), 1.83-1.91 (m, 2H), 2.04-2.1 (m, 2H), 2.15-2.18 (m, 1H), 2.52-2.60 (m, 2H), 2.95-3.01 (m, 2H), 3.22 (m, 1H), 3.93 (s, 3H, CH_3), 5.85 (s, 1H), 6.51 (s, 1H), 7.22 (d, J = 6.0 Hz, 1H), 7.24 (d, J = 6.0 Hz, 1H), 8.10 (d, J = 6.0 Hz, 1H), 8.12 (d, J = 6.2 Hz, 1H); MS (*m/z*, 100%) 383 ($[M+1]^+$, 100), 405 ($[M+23]^+$, 85).

Compound 2u: White solid, mp 114-115 ºC, yield: 92.5%, IR (KBr) ν 3032, 2951, 2924, 1712, 1662, 1618, 1566, 1450, 1375, 1339, 1240, 1145, 918 cm^{-1}; ^1H NMR (400 MHz, $CDCl_3$) δ 0.68 (d, J = 7.3 Hz, 3H, CH_3), 1.66 (s, 3H, CH_3), 1.69-1.87 (m, 4H), 2.05-2.11 (m, 1H), 2.14-2.16 (m, 1H), 2.43-2.51 (m, 1H), 2.59-2.65 (m, 1H), 2.85-2.98 (m, 1H), 3.20-3.21 (m, 1H), 4.06 (s, 6H, $2OCH_3$), 5.74 (s, 1H), 6.38 (s, 1H); MS (*m/z*, 100%) 387 ($[M]^+$, 100).

Biological Activity. The anti-viral activities of the synthesized compounds **2a-2u** were assayed against influenza virus A_3, B. Briefly, each compound for assaying was dissolved in DMSO at a concentration of 1000 μg/mL, then

diluted successively with 3-fold amount of nutrient mixture (composed with 10-15% fetal calf serum, 1% nonessential amino acid, and 0.66 mM L-glutamine) for 8 different concentrations (250, 62.5, 15.6, 3.9, 1.0, 0.2, 0.06 and 0.02 μg/mL respectively) as stock solution for the below experiment.

The Procedure for Anti-influenza Virus Assay. Madin-darby canine kidney cells (MDCK) were seeded in 96-well trays and cultured at 37 °C in a humidified CO_2 incubator (95% air, 5% CO_2) for 24 h, then which were infected with influenza virus A_3 with 1×10^{-3} [100-fold of the 50% tissue culture infective dose ($TCID_{50}$)] and with influenza B virus with 1×10^{-2} [30-fold of the 50% tissue culture infective dose ($TCID_{50}$)] respectively. All infected tissue culture plates (96 wells) were incubated at 37 °C for 2 h, medium was removed. Subsequently, 100 μL solution of different concentration of each compound was added to each well (every concentration was repeated for 3 times), the plates were incubated again for 36 h at 37 °C. Then the inhibition of virus-induced cytopathic effect (CPE) for each sample was recorded referring to the cell control and virus control according to the literature,[9,13] and 50% cell-inhibitory concentration (IC_{50}) values of active compounds were calculated accordingly. The inhibitory potentials of rupestonic acid derivatives were comparable to parent compound, the reference drug Ribavirin (RBV) and Oseltamivir.

Table 1. The results of the rupestonic acid ester derivatives **2a-2u** against influenza A_3 and B viruses

Compd.	[a]TC_{50} (μM)	Against influenza virus A_3		Against influenza virus B	
		[b]IC_{50} (μM)	[c]SI	[b]IC_{50} (μM)	[c]SI
[d]**1**	1044.4	–[e]	–[f]	115.7	9.0
2a	611.8	331.3	1.8	329.0	1.9
2b	193.6	39.4	4.9	50.9	3.8
2c	153.1	–[e]	–[f]	–[e]	–[f]
2d	1335.6	308.6	4.3	239.4	5.6
2e	621.9	–[e]	–[f]	112.3	5.5
2f	127.9	–[e]	–[f]	–[e]	–[f]
2g	706.1	–[e]	–[f]	–[e]	–[f]
2h	> 250	–[e]	–[f]	–[e]	–[f]
2i	138.3	–[e]	–[f]	–[e]	–[f]
2j	41.4	16.7	2.5	19.4	2.1
2k	191.3	13.4	14.3	–[e]	–[f]
2l	18.9	0.5	37.8	–[e]	–[f]
2m	141.5	43.5	3.3	–[e]	–[f]
2n	520.8	12.0	4.3	–[e]	–[f]
2o	240.5	11.5	12.6	–[e]	–[f]
2p	49.6	11.5	4.3	–[e]	–[f]
2q	136.3	–[e]	–[f]	–[e]	–[f]
2r	44.2	10.2	4.4	–[e]	–[f]
2s	189.5	29.4	6.4	24.9	7.6
2t	22.5	6.3	3.6	–[e]	–[f]
2u	370.0	–[e]	–[f]	–[e]	–[f]
Oseltamivir	> 1219.5	5.1	> 239.1	–[e]	–[f]
Ribavirin	1573.8	1.6	983.6	11.9	132.3

[a]50% cytotoxic concentration. [b]50% virus-inhibitory concentration, determined by CPE inhibition assay. [c]Selectivity Index (TC_{50}/IC_{50}). [d]Parent compound. [e]No anti-viral activity at the 50% cytotoxic concentration. [f]The SI can't be calculated,since the highest concentration tested was less than the IC_{50}.

Results and Discussion

In this study, we used the common coupling reagent DCC, HOBt/DMAP to activate the carboxyl group of rupestonic acid and synthesized the desired compounds **2a-2u**.

All synthesized compounds **2a-2u** were preliminarily screened *in vitro* against the strains of influenza A_3/Jifang/90/15 and B/Jifang/97/13 viruses (obtained from Beijing viral institute) by the national center for drug screening of China. Their inhibitory activity (IC_{50}) values are listed in Table 1, respectively. The inhibition of active compounds (possessing IC_{50} values) against influenza A_3 and B viruses at different concentrations were shown in Figure 1 and Figure 2, respectively. The inhibition of the more active compounds **2l** and **2t** against influenza A_3 at the comcentation from 0.02 to 0.2 μg/mL showed in Figure 3.

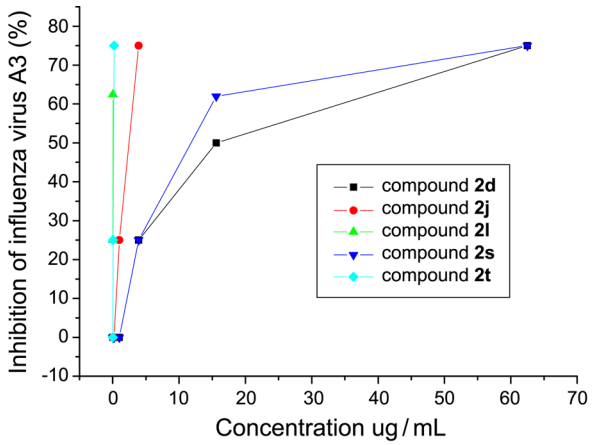


Figure 1. Inhibition and concentration-response curves of **2d**, **2j**, **2l**, **2s** and **2t** against influenza A_3 virus. Curves represent the average of three separate experiments.

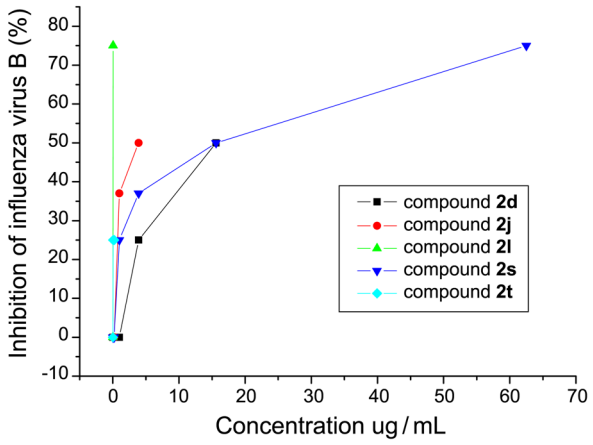


Figure 2. Inhibition and concentration-response curves of **2d**, **2j**, **2l**, **2s** and **2t** against influenza B virus. Curves represent the average of three separate experiments.

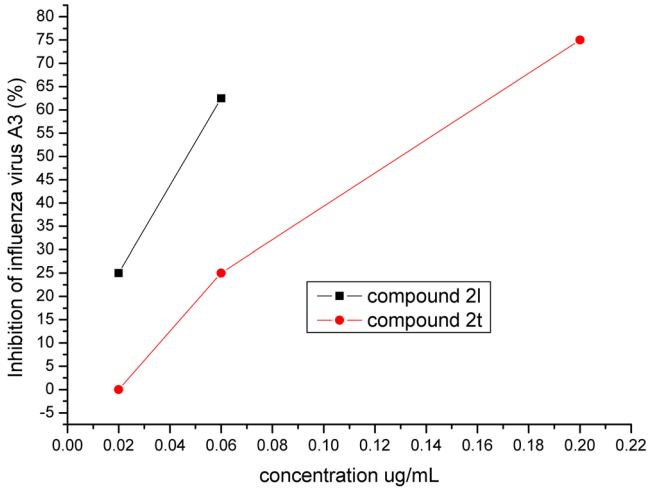


Figure 3. Inhibition and concentration-response curves of **2l** and **2t** against influenza A3 virus at the concentration from 0.02 to 0.2 μg/mL. Curves represent the average of three separate experiments.

As for the rupestonic acid fatty ester derivatives (**2a-2i**), the results showed that **2a**, **2b**, **2d** possess higher activities both against influenza virus A_3 and B, but the inhibition is not remarkable; As for the rupestonic acid aromatic ester derivatives (**2j-2u**), **2l** showed the highest inhibition against influenza A_3 virus (IC_{50} = 0.5 μmol/L), the inhibition was almost 10-fold higher than that of the reference drug Oseltamivir (IC_{50} = 5.1 μmol/L), the inhibition against influenza virus A_3 increased sharply and showed concentration-dependent at 0.02-0.06 μg/mL, the inhibition against influenza A_3 was 62.5% at 0.06 μg/mL. **2j** showed higher inhibition against both the strains of influenza A_3 (TC_{50} = 41.4 μmol/L, IC_{50} = 16.7 μmol/L, SI = 2.5) and B (TC_{50} = 41.4 μmol/L, IC_{50} = 19.4 μmol/L, SI = 2.1); **2s** also showed higher inhibition against both the strains of influenza A_3 (TC_{50} = 189.5 μmol/L, IC_{50} = 29.4 μmol/L, SI = 6.4) and B (TC_{50} = 189.5 μmol/L, IC_{50} = 24.9 μmol/L, SI = 7.6), the inhibition both to influenza A_3 and B viruses increased in concentration-dependent manner at a broad range of 0.02-62.5 μg/mL, the inhibitions both to influenza A_3 and B viruses at the highest concentration (62.5 μg/mL) was 75%; **2m**, **2n**, **2o**, **2p**, **2r**, **2t** all possess higher inhibition to influenza viral A_3 and **2t** (IC_{50} = 6.3 μmol/L) possess have almost the equivalent IC_{50} values to the Oseltamivir. As far as the IC_{50} values for anti-influenza virus A_3 among the rupestonic aromatic ester derivative (**2j-2u**) concerned, the active sequence is **2l** (IC_{50} = 0.5 μM) > **2t** (IC_{50} = 6.3 μM) > **2r** (IC_{50} = 10.2 μM) > **2o** (IC_{50} = 11.5 μM) = **2p** (IC_{50} = 11.5 μM) > **2n** (IC_{50} = 12.0 μM) > **2k** (IC_{50} = 13.4 μM) > **2j** (IC_{50} = 16.7 μM) > **2s** (IC_{50} = 29.4 μM) > **2m** (IC_{50} = 43.5 μM), which suggested that the inhibition against influenza virus A_3 will increase while introduction the big hydrophobic group at 4-position of phenyl ring; As for the compounds **2o-2r** (the phenyl ring substituted by halogen), there is not much difference of the IC_{50} values of the active compounds; **2t** showed higher activity against influenza A_3 virus and has almost the equivalent IC_{50} values to the Oseltamivir, we think that it owes to the skeleton of benzoic acid (the benzoic acid derivatives are also influenza neuraminidase inhibitors[14]), but unfortunately, the selective index value (SI = 3.6) is low and the toxicity is much higher than that of the Oseltamivir's. The Figure 3 showed that **2l** is more active against influenza A_3 than that of the compound **2t** at the same concentation.

In summary, we firstly synthesized a series of rupestonic acid ester derivatives and preliminarily evaluated for their antiviral activity against the strains of influenza A_3 and B viruses *in vitro*. On the whole, **2l** exhibited potent antiviral activity against the strains of influenza A_3 virus can be as a lead compound for anti-influenza A_3 virus. These results provided guidance for us and encouraged us to synthesize more rupestonic acid derivatives for the development of new anti-influenza viral drugs.

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