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Synthesis and Biological Evaluation of Gem-Difluoromethylenated Statin Derivatives as Highly Potent HMG-CoA Reductase Inhibitors

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HMG-CoA reductase inhibitors were widely used as lipid-lowing agents through effectively blocking the rate-limiting step of cholesterol biosynthesis. 8 analogs of Rosuvastatin were firstly prepared with different distance and functional group between the O5-hydroxyl group and terminal COOH group in the hydrophilic side-chain. In primary and secondary screening of the inhibitory activities against human HMG-CoA reductase, gem-difluoromethylenated derivatives exhibited more than 50% inhibition rate. Then 4 compounds with gem-difluoro group were further synthesized and evaluated in vitro, three compounds among them exhibited low single digital nmol/L IC_{50} values against HMG-CoA reductase. Molecular docking also well explained the observed special contribution of the gem-difluoro group.

Keywords HMG-CoA reductase inhibitors, gem-difluoromethylenated compounds, statin derivatives, hypercholesterolemia, molecular docking

Introduction

Coronary heart disease (CHD) is becoming a leading cause of death and keeps an increasing pressure on healthcare worldwide.[1] Numerous and diverse studies show that hypercholesterolemia is one of the key risk factors for the CHD.[2] In recent years, statins as potent 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase inhibitors were widely used as CHD drugs through effectively blocking the rate-limiting step of cholesterol biosynthesis.[3] Except marked HMG-CoA reductase inhibitors, such as Compactin, Pravastatin, Lovastatin, Simvastatin, Fluvastatin, Carivastatin, Atovastatin, Rosuvastatin, and Pitavastatin, several research groups including Mexico group, Zhou group, Nissan Chemical, BMS, and Pfizer have developed their novel HMG-CoA reductase inhibitors in recent years (Figure 1).[4] These HMG-CoA reductase inhibitors consisted of three parts: hydrophilic β,δ-dihydroxy-pentanoic acid unit or cyclized β-hydroxy-δ-lactone (red region in Figure 1), lipophilic aromatic core, and C—C or C=C linker (blue region in Figure 1). Although many modifications focused on the lipophilic cores and synthesis optimization on the hydrophilic side-chain[5] have been done, discovery of potent HMG-CoA reductase inhibitors with novel structure is still needed in medical community.

Since fluorine substituents affect nearly all physical and adsorption, distribution, metabolism, and excretion properties of a lead compound, fluorine substituents have become a widespread and important drug components.[6] The F substituent in the lipophilic pyrrole core of atorvastatin was found to be superior to ligands with hydroxyl, hydrogen, or methoxy.[6] After long focus on synthesis, especially on the synthesis of bioactive fluorine-containing compounds,[7] we estimated that F substituents in the hydrophilic side-chain of statin derivatives also cause positive effects on the enzymatic inhibitory activity. Herein, we would like to report the full discovery process including synthesis, *in vitro* biological evaluation, and molecular docking of the gem-difluoromethylenated statin derivatives as highly potent HMG-CoA reducatase inhibitors.

Experimental

Commercially available reagents and anhydrous solvents were used without further purification unless otherwise specified. Thin layer chromatography (TLC) analyses were performed with precoated silica gel.

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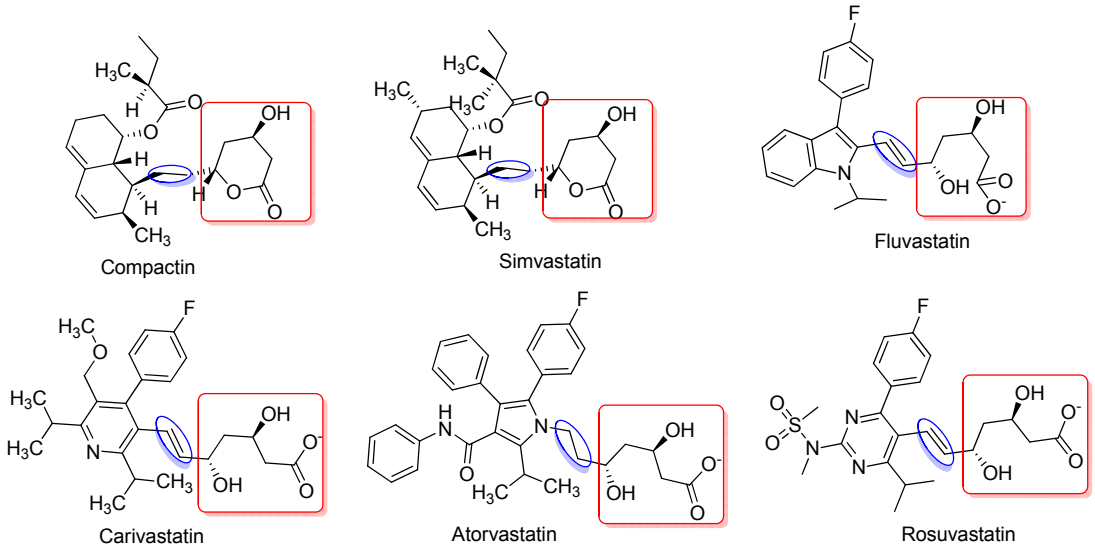

Figure 1 HMG-CoA reductase inhibitors.

Column chromatography was carried out on silica gel (300—400 mesh). All the products were purified by column chromatography on silica gel with ethyl acetate-petroleum ether in an appropriate ratio as the eluent. All compounds were determined to be >95% pure by prepared HPLC. NMR spectra were recorded with a Bruker® 400 MHz spectrometer or Bruker® 500 MHz spectrometer at ambient temperature with the residual solvent peaks as internal standards. IR spectra (KBr) were recorded on a Nicolet In10 FT-IR spectrometer in the range of 400—4000 cm^{-1}. The melting points were measured on an SGW X-4 melting point instrument. The HRMS spectra were acquired on a Solaril X70 FT-MS apparatus or Advion expression CMS apparatus. **10d** was obtained as a gift from Dr. Wang.[7a]

Synthetic procedures of inhibitors 2—4, 6—9, and 10a—10c

$NaBH_4$ (0.5 mmol) was added dropwise to a solution of *N*-[4-(4-fluorophenyl)-6-isopropyl-5-(3-oxopropenyl)-pyrimidin-2-yl]-*N*-methyl-methanesulfonamide **1** (1 mmol, 120.78 mg) in THF (5 mL) and MeOH (3 mL) under –45 ℃. The reaction mixture was stirred for another 2 h at –45 ℃, and then poured into a saturated $NaHCO_3$ solution. The aqueous layer was extracted with ethyl acetate and the combined organic phase was washed with brine and dried over anhydrous Na_2SO_4. After concentrated in vacuum, the residue was purified by column chromatography on silica gel (petroleum *V*(ether) ∶ *V*(ethyl acetate) = 4 ∶ 1) to provide compound **2** as a yellow liquid in 41% yield.

(*E*)-*N*-(4-(4-Fluorophenyl)-5-(3-hydroxyprop-1-en-1-yl)-6-isopropylpyrimidin-2-yl)-*N*-methylmethanesulfonamide (2) ^1H NMR (400 MHz, $CDCl_3$) δ: 7.67—7.64 (m, 2H), 7.12—7.08 (m, 2H), 6.61—6.57 (m, 1H), 5.69—5.63 (m, 1H), 4.22—4.21 (m, 2H), 3.57 (s, 3H), 3.52 (s, 3H), 3.43—3.37 (m, 1H), 1.27 (s, 6H); IR (KBr) *v*: 3443, 2925, 2852, 2026, 1906, 1633, 1401, 1137, 1125, 965, 607, 566 cm^{-1}; HRMS calcd for $C_{18}H_{23}FN_3O_3S$ $[M+H]^+$: 380.1444, found 380.1433.

Ethyl 2-bromo-2,2-difluoroacetate (1.89 mmol) was added slowly at room temperature to a stirred mixture of THF (2.5 mL) and freshly activated zinc powder (1.89 mmol). Then, a solution of acrylaldehyde **1** (0.63 mmol) in THF (3 mL) was added dropwisely. After completion of the addition, the reaction mixture was refluxed for 5 h, and then quenched with saturated ammonium chloride. The excess zinc powder was removed by suction filtration and the filter cake was washed with ethyl acetate. The combined filtrate was extracted with ethyl acetate, washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuum. The remaining residue was purified by column chromatography on silica gel [*V*(petroleum ether) ∶ *V*(ethyl acetate) = 5 ∶ 1] to produce **3** as a yellow solid in 60% yield.

(*E*)-2,2-Difluoro-5-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethylsulfonamido)pyrimidin-5-yl)-3-hydroxypent-4-enoate (3) m.p. 170—171 ℃; ^1H NMR (400 MHz, $CDCl_3$) δ: 7.68—7.63 (m, 2H), 7.13—7.08 (m, 2H), 6.80 (d, *J* = 16.0 Hz, 1H), 5.64 (d, *J* = 16.0 Hz, 1H), 4.71—4.64 (m, 1H), 4.34 (q, *J* = 6.4 Hz, 2H), 3.58 (s, 3H), 3.52 (s, 3H), 3.40—3.34 (m, 1H), 1.36 (t, *J* = 6.4 Hz, 3H), 1.29—1.26 (m, 6H); ^{19}F NMR (471 MHz, $CDCl_3$) δ: –106.53 (s, 1F), –107.10 (s, 1F), –111.63 (s, 1F); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 179.7, 168.0, 164.2, 158.0, 134.4, 130.7, 116.1, 115.7, 115.5, 77.3, 77.0, 76.8, 69.7, 63.5, 42.6, 33.5, 33.1, 23.7, 21.7, 13.8; IR (KBr) *v*: 3475, 2966, 2926, 2850, 1759, 1605, 1548, 1510, 1439, 1338, 1231, 1154, 1079, 962, 844, 773, 575, 518 cm^{-1}; HRMS calcd for $C_{22}H_{27}F_3N_3O_5S$ $[M+H]^+$: 502.1624, found 502.1650.

NaOH (1.125 mmol) was added slowly to a solution of ester **3** (0.225 mmol) in methanol (2 mL) at room temperature. The resulting mixture was stirred for 2 h. Then the reaction mixture was concentrated to remove methanol, extracted with ethyl acetate. The aqueous

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layer was washed with 10% HCl, extracted a second time with ethyl acetate, washed with brine, dried over anhydrous sodium sulfate, and concentrated in vacuum to give a residue. The residue was purified by column chromatography on silica gel [*V*(petroleum ether)∶*V*(ethyl acetate)=1∶1] to give **4** as a yellow oil in 59% yield.

(*E*)-2,2-Difluoro-5-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethylsulfonamido)pyrimidin-5-yl)-3-hydroxypent-4-enoic acid (4) ^{1}H NMR (DMSO-d_6, 400 MHz) δ: 7.77−7.73 (m, 2H), 7.28−7.24 (m, 2H), 6.67−6.60 (m, 1H), 6.36 (br, 1H, OH), 5.86−5.60 (m, 1H), 4.40−4.34 (m, 1H), 3.54 (s, 3H), 3.45 (s, 3H), 1.23−1.19 (m, 6H); IR (KBr) *v*: 3412, 2967, 2929, 1648, 1604, 1545, 1509, 1437, 1382, 1337, 1229, 1154, 1068, 965, 814, 775, 576, 566, 520 cm^{-1}; HRMS calcd for $C_{20}H_{23}F_3N_3O_5S$ $[M+H]^+$: 474.1311, found 474.1350.

Keeping the inner temperature at −78 ℃, *n*-butyllithium (2.2 mol/L, 10.9 mL) was added to a solution of diisopropylethylamine (3.4 mL, 24 mmol) in THF (5 mL) over 20 min under N_2 atmosphere. The reaction mixture was stirred at −30 to −40 ℃ for 30 min. *tert*-Butyl acetate (6.5 mL, 24 mmol) was added over 10 min. The reaction mixture was stirred for 30 min. Then the mixture was cooled to −78 ℃, and a solution of **3** (8 mmol) in THF was added over 30 min. The reaction mixture was stirred for 2 h, and then the temperature was raised to 0 ℃ over 30 min. The reaction was quenched with saturated aqueous ammonium chloride solution and stirred for 15 min. The aqueous layer was extracted with ethyl acetate and the combined organic layer was washed with brine and dried over anhydrous sodium sulfate. After evaporation of the solvent, the residue **5** was used for the next step without further purification.

Following the synthesis procedure of **2** from **1**, **6** was obtained from **5** as a yellow solid in 50% yield after two steps.

(*E*)-*tert*-Butyl-4,4-difluoro-7-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethylsulfonamido)pyrimidin-5-yl)-3,5-dihydroxyhept-6-enoate (6) m.p. 111−112 ℃; ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.69−7.61 (m, 2H), 7.11−7.07 (m, 2H), 6.82 (d, *J*=16.0 Hz,1H), 5.69 (d, *J*=16.0 Hz, 1H), 4.64 (s, 1H), 3.57 (s, 3H), 3.52 (s, 3H), 3.38−3.34 (m, 1H), 2.62−2.58 (m, 2H),1.48 (s, 9H), 1.28 (d, *J*=3.3 Hz, 3H), 1.27 (d, *J*=3.3 Hz, 3H); ^{19}F NMR (471 MHz, $CDCl_3$) δ: −110.86 (s, 1F), −117.91 (d, 1F), −127.55 (d, 1F); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 175.0, 171.9, 163.7, 157.7, 134.0, 132.2, 128.0, 120.9, 115.3, 82.5, 77.3, 42.4, 34.4, 34.1, 33.1, 32.1, 29.5, 28.0, 21.7, 14.3, 1.0; IR (KBr) *v*: 3442, 2981, 2934, 1716, 1604, 1556, 1509, 1445, 1401, 1383, 1197, 1156, 1096, 1066, 905, 840, 756, 567, 519 cm^{-1}; HRMS calcd for $C_{26}H_{35}F_3N_3O_6S$ $[M+H]^+$: 574.2199, found 574.2181.

To a mixture of DAST (3.78 mmol) in THF (10 mL) at −78 ℃, a solution of **6**（1.26 mmol）in THF (10 mL) was added slowly. The mixture was stirred at −78 ℃ for 5 h, at 0 ℃ for 1 h, and at room temperature for 1 h. The reaction mixture was then poured into saturated aqueous $NaHCO_3$. The mixture was extracted with ethyl acetate. The combined organic phase was washed with water, dried over anhydrous sodium sulfate and evaporated under reduced pressure to give an oil residue. The oil was purified by column chromatography on silica gel [*V*(petroleum ether)∶*V*(ethyl acetate)=5∶1) to provide **7** as a yellow oil in 21% yield.

(*E*)-*tert*-Butyl-4,4,5-trifluoro-7-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethylsulfonamido)pyrimidin-5-yl)-3-hydroxyhept-6-enoate (7) ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.55 (d, *J*=8.5 Hz, 2H), 7.20 (d, *J*=8.5 Hz, 2H), 6.49−6.38 (m, 1H), 6.31−6.17 (m, 1H), 5.98−5.86 (m, 1H), 4.19 (s, 1H), 3.60 (s, 3H), 3.54 (s, 3H), 3.48−3.35 (m, 1H), 2.67−2.44 (m, 2H), 1.49 (s, 9H), 1.32−1.29 (m, 6H); ^{19}F NMR (471 MHz, $CDCl_3$) δ: −105.28−−106.99 (m, 1F), −112.01−−113.75 (m, 2F), −170.69−−173.69 (m, 1F); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 178.6, 166.6, 163.9, 158.6, 137.1, 134.5, 131.2, 127.5, 116.6, 87.9, 86.5, 81.9, 60.4, 42.5, 32.9, 27.9, 22.7, 21.4, 21.0, 14.2, 1.0; IR (KBr) *v*: 3359, 3192, 2955, 2922, 2851, 1659, 1632, 1548, 1510, 1466, 1156, 962, 668, 649 cm^{-1}; HRMS calcd for $C_{26}H_{34}F_4N_3O_5S$ $[M+H]^+$: 576.2155, found 576.2150.

TFA (15 mmol) was added to a solution of ester **7** (0.5 mmol) in dichloromethane (15 mL), and the mixture was stirred at 0 ℃ for 1 h，and at room temperature for 12 h. The mixture was then poured into a stirring mixture of saturated aqueous $NaHCO_3$. The mixture was extracted with dichloromethane. The organic phase was washed with brine, dried over anhydrous sodium sulfate and evaporated under reduced pressure to give an oil residue. The residue was purified by column chromatography on silica gel [*V*(petroleum ether)∶*V*(ethyl acetate)=1∶1) to provide **8** as a yellow oil in 46% yield.

(*E*)-*N*-(5-(2-(3,3-Difluoro-4-hydroxy-6-oxotetrahydro-2*H*-pyran-2-yl)vinyl)-4-(4-fluorophenyl)-6-isopropylpyrimidin-2-yl)-*N*-methylmethanesulfonamide (8) ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.67 (d, *J*=8.6 Hz, 2H), 7.12 (d, *J*=8.6 Hz, 2H), 6.86 (d, *J*=16.2 Hz, 1H), 5.66 (d, *J*=16.2 Hz, 1H), 5.38−5.28 (m, 1H), 4.29 (s, 1H), 3.60 (s, 3H), 3.54 (s, 3H), 3.44−3.35 (m, 1H), 3.07−2.89 (m, 2H), 1.29−1.26 (m, 6H); ^{19}F NMR (471 MHz, $CDCl_3$) δ: −111.11 (s, 1F), −123.91 (s, 1F), −124.10 (s, 1F); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 174.8, 172.5, 167.4, 163.3, 157.5, 132.6, 126.6, 121.7, 115.6, 79.7, 79.2, 42.0, 33.6, 32.1, 31.7, 29.5, 21.9, 21.7, 14.5; IR (KBr) *v*: 3467, 2964, 2928, 1712, 1605, 1546, 1509, 1439, 1381, 1335, 1230, 1155, 1066, 963, 845, 576, 520 cm^{-1}; HRMS calcd for $C_{22}H_{25}F_3N_3O_5S$ $[M+H]^+$: 500.1467, found 500.1461.

Following the synthesis procedure of **7** from **6**, **9** was obtained from **8** as a yellow oil in 22%.

(*R*,*E*)-*N*-(5-(2-(4,4-Difluoro-7-oxo-2,3,4,7-tetrahydro-oxepin-3-yl)vinyl)-4-(4-fluorophenyl)-6-isopropylpyrimidin-2-yl)-*N*-methylmethane sulfonamide (9) ^{1}H NMR (500 MHz, $CDCl_3$) δ: 7.70−7.60 (m, 2H), 7.17−7.07 (m, 2H), 6.94−6.90 (m, 1H), 6.87−6.83 (m, 1H), 6.36−6.34 (m, 1H), 5.72−5.68

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(m, 1H), 5.14－5.06 (m, 1H), 4.22－4.01 (m, 2H), 3.60 (s, 3H), 3.54 (s, 3H), 3.42－3.36 (m, 1H), 1.30 (d, J= 6.3 Hz, 6H); ^{19}F NMR (471 MHz, $CDCl_3$) δ: −106.51 (s, 1F), −108.65 (s, 1F), −110.78 (s, 1F); ^{13}C NMR (125 MHz, $CDCl_3$) δ: 175.0, 171.1, 163.9, 157.8, 137.9, 132.1, 125.1, 119.8, 115.5, 79.7, 60.4, 42.4, 33.1, 32.2, 29.7, 23.4, 21.7, 21.0, 14.1; IR (KBr) v: 2961, 2925, 2854, 1750, 1605, 1547, 1509, 1439, 1382, 1338, 1229, 1156, 1106, 1073, 962, 845, 815, 772, 575, 520 cm^{-1}. HRMS calcd for $C_{22}H_{23}F_3N_3O_4S$ $[M+H]^+$: 482.1356, found 482.1354.

Following the synthesis procedure of **4** from **3**, **10a** was obtained from **6** as a yellow oil in 49% yield.

(*E*)-4,4-Difluoro-7-(4-(4-fluorophenyl)-6-isopropyl-2-(*N*-methylmethylsulfonamido)pyrimidin-5-yl)-3,5-dihydroxyhept-6-enoic acid (10a) ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.76－7.71 (m, 2H), 7.29－7.24 (m, 2H), 6.74 (d, J=16.0 Hz, 1H), 5.65 (d, J=16.0 Hz, 1H), 4.55－4.43 (m, 1H), 4.16－4.01 (m, 1H), 3.55 (s, 3H), 3.45 (s, 3H), 2.58－2.54 (m, 1H), 2.36－2.30 (m, 1H), 1.23－1.21 (m, 6H); ^{19}F NMR (471 MHz, DMSO-d_6) δ: –111.53－–111.92 (m, 1F), –118.87 (d, J=250.6 Hz, 1F), –122.38 (d, J=250.6 Hz, 1F); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 174.9, 163.3, 162.1, 157.5, 134.6, 132.6, 126.9, 121.7, 115.5, 70.4, 67.3, 60.2, 55.1, 42.0, 36.5, 33.6, 31.8, 21.9, 14.1; IR (KBr) v: 3466, 2969, 2932, 2872, 2389, 2251, 1711, 1605, 1545, 1510, 1438, 1381, 1333, 1231, 1155, 1067, 964, 901, 845, 777, 576, 566, 520 cm^{-1}; HRMS calcd for $C_{22}H_{27}F_3N_3O_6S$ $[M+H]^+$: 518.1573, found 518.1565.

Following the synthesis procedure of **10a** from **1**, **10b** was obtained from 3-[3-(4-fluorophenyl)-1-isopropyl-1*H*-indol-2-yl]-propenal as a yellow oil in 11% yield after 4 steps.

7-[2-Cyclopropyl-4-(4-fluorophenyl)-quinolin-3-yl]-4,4-difluoro-3,5-dihydroxyhept-6-enoic acid (10b) ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.68－7.66 (m, 1H), 7.45－7.42 (m, 3H), 7.25－7.21 (m, 2H), 7.18－7.14 (m, 1H), 7.06－7.02 (m, 1H), 6.86－6.82 (m, 1H,), 5.97－5.90 (m, 1H), 5.83－5.77 (m, 2H), 4.95－4.88 (m, 1H), 4.56－4.45 (m, 1H), 4.18－4.10 (m, 1H), 2.60 －2.58 (m, 1H), 2.38－2.24 (m, 1H), 1.68－1.65 (m, 6H); ^{19}F NMR (471 MHz, DMSO-d_6) δ: −113.86 (s, 1F), –123.43 － −123.54 (m, 2F); ^{13}C NMR (125 MHz, DMSO) δ: 172.6, 162.1, 160.2, 135.4, 133.6, 132.1, 127.9, 122.3, 120.1, 119.3, 115.9, 114.0, 112.5, 69.3, 66.0, 60.2, 56.3, 47.6, 35.9, 21.5, 14.5; IR (KBr) v: 3392, 3068, 2927, 2855, 1709, 1636, 1574, 1513, 1489, 1411, 1365, 1261, 1158, 1093, 1057, 1023, 932, 841, 765, 619, 558, 530 cm^{-1}; HRMS calcd for $C_{24}H_{25}F_3NO_4$ $[M+H]^+$: 448.1736, found 448.1731.

Following the synthesis procedure of **10a** from **1**, **10c** was obtained from 3-[2-cyclopropyl-4-(4-fluorophenyl)-quinolin-3-yl]-propenal as a yellow oil in 15% yield after 4 steps.

(*E*)-4,4-Difluoro-7-[3-(4-fluorophenyl)-1-isopropyl-1*H*-indol-2-yl]-3,5-dihydroxyhept-6-enoic acid (10c) ^{1}H NMR (500 MHz, DMSO-d_6) δ: 7.85－7.86 (m, 1H), 7.68－7.67 (m, 1H), 7.45－7.44 (m, 1H), 7.31 －7.39 (m, 4H), 7.25－7.27 (m, 1H), 6.73－6.67 (m, 1H), 5.82－5.74 (m, 1H), 4.43－4.27 (m, 2H), 4.11－4.03 (m, 1H), 2.53－2.33 (m, 1H), 2.33－2.23 (m, 1H), 2.15－2.07 (m, 1H), 1.25－1.23 (m, 2H), 1.03－1.01 (m, 2H); ^{19}F NMR (471 MHz, DMSO-d_6) δ: –116.59 (s, 1F), –116.70 (s, 1F), –123.07 (s, 1F); ^{13}C NMR (125 MHz, DMSO-d_6) δ: 175.4, 163.9, 161.9, 147.1, 131.6, 131.3, 130.9, 130.8, 130.3, 128.3, 126.7, 126.1, 125.8, 116.1, 90.1, 88.8, 60.6, 29.7, 21.0, 16.1, 14.2, 10.7; IR (KBr) v: 3362, 2977, 2851, 1887, 1659, 1500, 1456, 1402, 1437, 1347, 1107, 1095, 999, 971, 941, 839, 815, 739, 564, 523 cm^{-1}. HRMS calcd for $C_{25}H_{23}F_3NO_4$ $[M+H]^+$: 458.1579, found 458.1571.

Biological evaluation *in vitro*[10]

All inhibitors were dissolved with NaOH/THF (1/1) at 0.1 mol/L concentration and stored in a –20 ℃ fridge. Then statin inhibitors were diluted with the buffer to the concentration of 1×10^{-4}, 1×10^{-5}, 1×10^{-6}, 1×10^{-7}, or 1×10^{-8} mol/L. The spectrophotometer was seated at 37 ℃ and 340 nm, with a kinetic program. Then 181 μL assay buffer, 1 μL inhibitor or pravastatin, 4 μL NADPH, 12 μL HMG-CoA, and HMG-CoA reductase were added to the well, respectively. Then the samples mixture was mixed thoroughly and the kinetics program was started immediately. The enzymatic reaction was initiated by the addition of HMG-CoA. The UV absorbance was measured twice for each sample. The inhibitory rate values were worked out by the equation in Figure 2. The IC_{50} values were worked out through graphpad prism 5 nonlinear regression calculation.

$$\text{Units/mgP} = \frac{(\Delta A_{340}/\text{min}_{\text{sampl}} - \Delta A_{340}/\text{min}_{\text{blank}}) \times V_T}{12.44 \times V \times 0.6 \times LP}$$

$$\text{Inhibition rate} = \frac{\text{Units/mgP}_{\text{negative}} - \text{Units/mgP}_{\text{sample}}}{\text{Units/mgP}_{\text{negative}}} \times 100\%$$

ΔA_{340} = Change value of absorbance at 340 nmol/L
V_T = Total volume of the reaction in mL
(1 mL for cuvettes and 0.2 mL for plates)
V = volume of enzyme used in the assay (mL)
LP = Light path in cm (1 for cuvettes and 0.55 for plates)
Units/mgP: the unit specific activity [μmol/(min•mg protein)]
Inhibition rate: the ability that the inhibitors inhibit the enzyme activity
$\text{Units/mgP}_{\text{negative}}$: unit specific activity when HMGR exsists in the reaction system without inhibitors.

Figure 2 Formula of inhibition rate.

Molecular docking

The ligand preparation was performed using SYBYL 2.0. Energy minimization was optimized using Tripos molecular mechanics force field with energy gradient criterion of 0.005 kcal (Å•mol), and the atom charges of Tripos force field were calculated by the Gasteiger-Huckel method. The crystal structures of HMGR for molecular docking were downloaded from RCSB Protein Data Bank (http:/www.rcsb.org/pdb/home/home.do, PDB: 2Q1L). All water molecules were removed and hydrogen atoms were added. In the docking process, the protein was fixed while the ligands were flexible, the

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ligands were docked into the ATP-binding site of HMGR by the Surflex-Dock module and the docked complex was evaluated by empirical scoring function. By default, 30 conformations were generated because each ligand and their scores were forecasted based on the strength of receptor-ligand interactions.

Results and Discussion

Chemistry

The synthesis of target compounds **2**—**4**, **6**—**9**, and **10a**—**10d** was presented in Scheme 1. Commercial available 3-substituted propenals **1** was reduced to 3-substituted allylic alcohol (**2**) by sodium borohydride in methanol. Zinc-mediated Reformatsky reaction of **1** with ethyl bromodifluoroacetate gave the ester **3**. Subsequent ester **3** was hydrolyzed to acid **4** with aqueous sodium hydroxide in ethanol. Treatment of ester **3** with acetic acid *tert*-butyl ester and lithium diisopropylamide (LDA) gave the *δ*-hydroxy-*β*-keto ester (**5**). Reduction of carbonyl group afforded the *β*,*δ*-diols *tert*-butyl ester (**6**). Compound **6** was converted into the *δ*,*γ*,*γ*-trifluoro ester (**7**) by the treatment with diethylamido sulfur trifluoride (DAST). To our expectation, compound **7** underwent cyclization to give lactone **8** in the presence of TFA in CH_2Cl_2, and no hydrolysis product was obtained. Reaction of compound **8** and DAST gave the hydroxyl elimination product **9**. Finally, *β*,*δ*-diol-ester (**6**) was hydrolyzed by sodium hydroxide to produce compound **10**.

Bioassay

In this study, all the synthesized compounds were evaluated for their ability to inhibit human HMG-CoA reductase. The assay is based on the spectrophotometric measurement of the decrease in absorbance at 340 nm, which represents the oxidation of NADPH by the catalytic subunit of HMG-CoA reductase in the presence of the substrate of HMG-CoA. The test was performed by using a spectrophotometric method according to a commercial HMG-CoA reductase assay kit (Sigma-Aldrich).

Rosuvastatin was chosen as the lead compound for our F-substituted modifications for it has the best IC_{50} value among those listed drugs in Figure 1.[8] The distance and functional group between the O5-hydroxyl group and the terminal COOH group were first studied. Eight compounds (**2**—**4**, **6**—**9**, and **10a**) with different chain lengths and functional groups were firstly synthesized and submitted to the preliminary biological evaluation *in vitro*. All of them exhibited inhibitory activities against HMG-CoA reductase at the concentration of 5×10^{-6} mol/L (Table 1). The inhibition rates were 44% for **2** with CH_2OH as the side-chain, 21% for **3** with *γ*-hydroxy-*β*,*β*-difluoro-ethyl ester as the side-chain, 21% for **4** with *γ*-hydroxy-*β*,*β*-difluoropropionic acid as the side-chain, 77% for **6** with *β*,*δ*-diols-*γ*,*γ*-difluoro-*tert*-butyl ester as the side-chain, 50% for **7** with *δ*,*γ*,*γ*-trifluoro-*β*-hydroxyl-*tert*-butyl ester as the side-chain, 59% for **8** with *γ*,*γ*-difluro-*β*-hydroxy-*δ*-lactone as the tail, 88% for **9** with *γ*,*γ*-difluro-*α*,*β*-unsaturated-*δ*-lactone as the side-chain, 80% for **10a** with *β*,*δ*-dihydroxy-*γ*,*γ*-difluoropentanoic acid as the side-chain, respectively. Shorting the chain length from 3 to 1 methylene unit obviously decreased the activity (Entries 4, 5 and 8 vs. Entries 2 and 3). Although all of them (Ester **6**, eliminated lactone **8**, and acid **10a)** exhibited good inhibitory activities for the enzymatic hydrolysis to carboxylate anion, the inhibition rates between them were different due to the hydrolysis degree and **10a** had the highest inhibition rate among them (Entry 8 vs. Entries 4 and 6). The replacement of methylene unit to gem-difluoromethylene slightly increased the inhibition rate from 77% to 80% (Entry 8 vs. Entry 9). However, the replacement of O5-hydroxy to fluoro caused lower inhibition rate (Entry 4 vs. Entry 5). It should be pointed out that compounds (**2**—**4**, **6**—**9**, and **10a**) were all racemates. Five compounds (**6**—**9**, and **10a**) exhibited more than 50% inhibition rate at the concentration of 5×10^{-6} mol/L, therefore they were diluted to lower concentration and submitted to second round *in vitro* biological evaluation.

At the concentration of 5×10^{-7} mol/L, all com-

Scheme 1 Reagents and condition: (a) $NaBH_4$, MeOH, 0 ℃; (b) $BrCF_2COOEt$, Zn, THF, reflux; (c) 1 equiv. NaOH, MeOH; then 2 equiv. HCl; (d) acetic acid *tert*-butyl ester, LDA, −78 ℃; (e) DAST, −78 ℃; (f) TFA, DCM

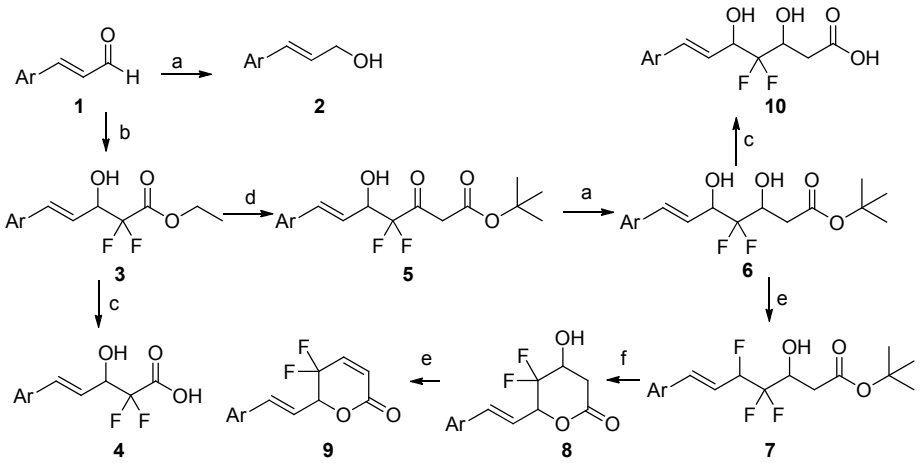

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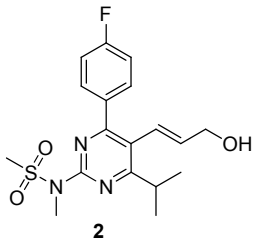
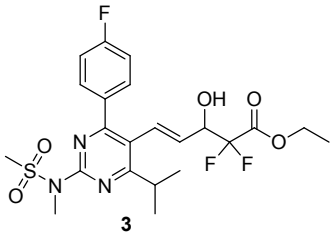
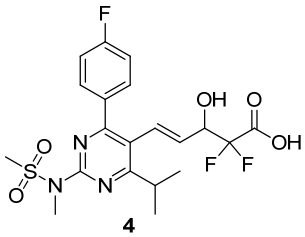
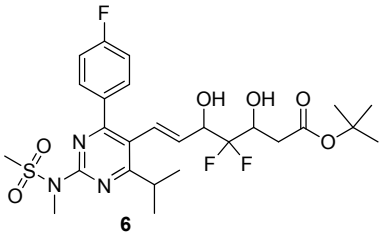
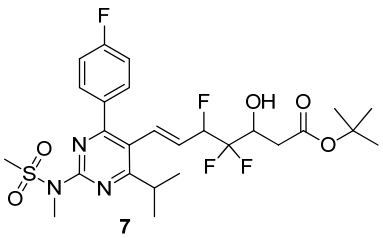
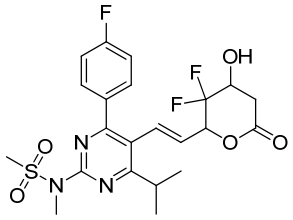
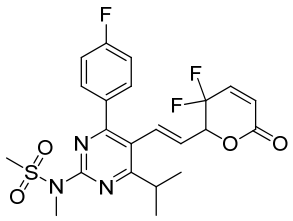
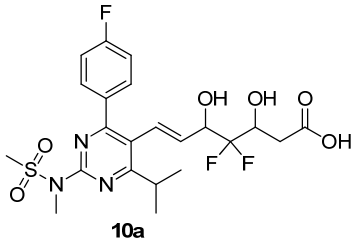
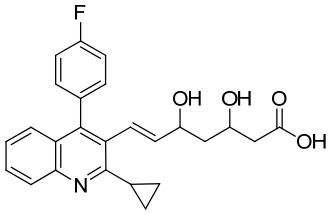
Table1 Inhibition rates

Entry	Compound	Inhibition rate[a]/%
1	 **2**	44
2	 **3**	21
3	 **4**	21
4	 **6**	77
5	 **7**	50
6	 **8**	59
7	 **9**	88

Continued

Entry	Compound	Inhibition rate[a]/%
8	 **10a**	80
9	 pitavastatin	77

[a] Inhibition rates were means of three or more experiments with errors within 30% of them, and the concentration was 5×10^{-6} mol/L.

pounds (**6**—**9**, **10a**, and pitavastatin) exhibited lower inhibitory activities compared with the concentration of 5×10^{-6} mol/L, and the inhibition rates order from high to low was 72% for inhibitor **10a**, 70% for inhibitor **6**, 62% for pitavastatin, 46% for inhibitor **7**, 40% for inhibitor **9**, and 29% for inhibitor **8** (Table 2). Again gem-difluoromethylenated ester **6** exhibited the similar good inhibition rate as acid **10a** for ester was easy to hydrolyze to carboxylate anion under test conditions (Entry 1 vs. 5), however gem-difluoromethylenated lactone **8** and **9** showed ＜50% inhibition rates probable due to the hydrolysis was too lower compared with ester (Entries 3 and 4 vs. Entry1). Trifluoro-containing inhibitor **7** also showed ＜50% inhibition rates (Entry 2).

Table 2 Inhibition rates

Entry	Compound	Inhibition rate[a]/%
1	**6**	70
2	**7**	46
3	**8**	29
4	**9**	40
5	**10**[a]	72
6	pitavastatin	62

[a] Inhibition rates were means of three or more experiments with errors within 30% of them, and the concentration was 5×10^{-7} mol/L.

Since **10a** displayed the best inhibition rate in both primary and second screening, it was treated as the new lead compound, and the modification on the lipophilic region of **10a** was then investigated (Table 3). For the

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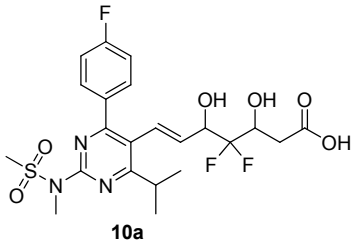
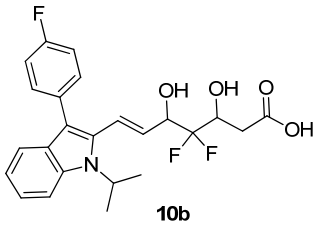
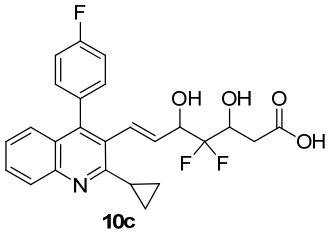
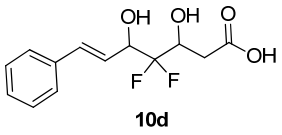
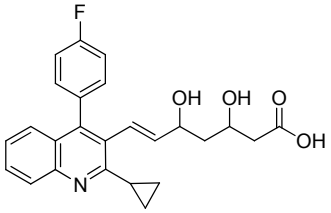
convenience of the synthesis, the preparation of gem-difluoro-substituted inhibitors **10a**—**10d** was started from commercial available 3-substituted (*E*)-propenals. Rosuvastatin derivative **10a**, fluvastatin derivative **10b**, and pitavastatin derivative **10c** had a little bit better inhibitory activities against HMG-CoA reductase compared with pitavastatin (IC_{50}=2.8 nmol/L for **10a**, 3.1 nmol/L for **10b**, and 4.7 nmol/L for **10c** vs. 8.4 nmol/L for pitavastatin), however the simplification of the lipophilic part to phenyl ring caused the totally lost of the inhibitory activities in the test window, probably due to the missing of the interaction between 4-fluorophenyl group and enzyme, and polar interaction between the arginine *ε*-nitrogen atoms and the fluorine atoms (Entries 4—5).[8]

Table 3 IC_{50} values

Entry	Compound	IC_{50}[a]/(nmol•L^{-1})
1	 **10a**	2.8
2	 **10b**	3.1
3	 **10c**	4.7
4	 **10d**	NR[b]
5	 pitavastatin	8.4

[a] IC_{50} values were means of three or more experiments with errors within 30% of them, and compounds were tested at five concentrations (5×10^{-6}, 5×10^{-7}, 5×10^{-8}, 5×10^{-9}, and 5×10^{-10} mol/L). [b] Out of the test window.

According to the structure-activity relationship (SAR) investigation of compounds **2**—**4**, **6**—**9**, and **10a**—**10d**, several informations were concluded that keeping chain length as 3 methylene units between O5-hydroxyl group and the terminal COOH group was necessary for the potency, gem-difluoro modification at *γ*-position of the hydrophilic side-chain benefited the inhibitory activity, the replacement of O5-hydroxyl by F generally decreased the activity, compounds with terminal COOH group had higher inhibition rate than esters or lactones, and the interaction between 4-fluoro-phenyl group in lipophilic region and enzyme was one of the key factors for the high potency of inhibitors.

Molecular docking

Computational approaches have aided our drug discovery process for a long time.[9] Molecular docking, which is widely used to explore the binding affinities of ligands, was used to guide and better explain the SAR for this series of gem-difluoromethylenated statin derivatives as highly potent HMG-CoA reductase inhibitors. **10a** as the most active inhibitor was docked into the catalytic domain of human HMG-CoA reductase (PDB: 2Q1L). As shown in Figure 3, the binding motif of (3*R*,5*S*)-**10a** revealed 9 H-bond interactions were formed between ligand and protein: Lys691 and gem-

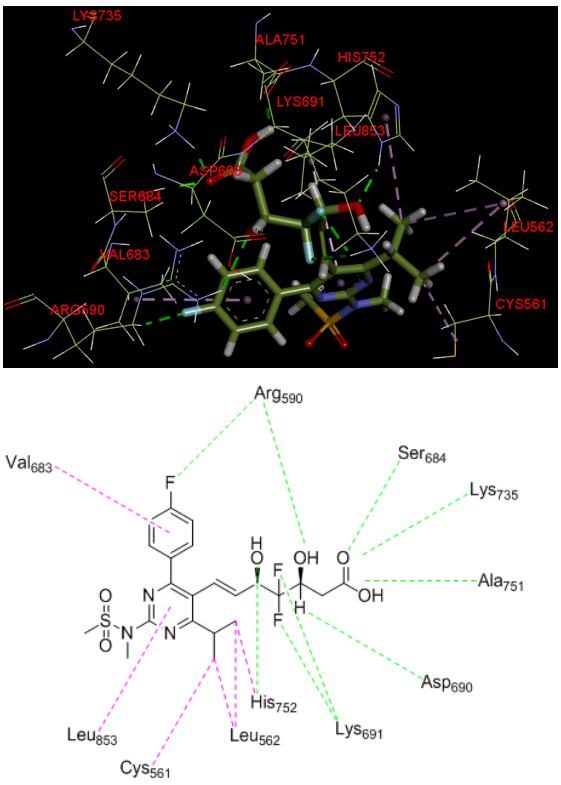


Figure 3 Docking results of (3*R*,5*S*)-**10a**. Hydrogen interactions were shown as green lines, and hydrophobic interactions were shown as pink lines.

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difluoro group at the side-chain, Arg590 and the fluorine atom in the lipophlic region, Arg590 and O3-hydroxyl group, His752 and O5-hydroxyl group, Lys735/Ser684 and carbonyl group, Ala751 and terminal OH, Asp690 amd H of C3. In addition to the H-bond interactions, an important contributor to the high potency of **10a** was the hydrophobic interactions. 6 hydrophobic interactions were formed: the 4-fluorophenyl moiety and Val683, isopropyl group and His752/Leu562/Cys561, pyrimidine moiety and Leu853. Similar H-bond interactions were also observed between protein and the gem-difluoro group in **10b** and **10c**. So the docking results well explained the observed SAR that the introduction of gem-difluoromethylenated moiety at the side chain contributed the HMG-CoA reductase inhibitory activity. Meanwhile, the presence of O5-hydroxyl group was essential.

Conclusions

In this work, 11 compounds were synthesized and evaluated against human HMG-CoA reductase. The obtained SAR demonstrated that gem-difluoro group on the hydrophobic side-chain of the statin derivatives increased the inhibition efficiency against the HMG-CoA reductase. Molecular modelling studies indicated that gem-difluoromethylenated Rosuvastatin derivative **10a** had H-bond interaction between the gem-difluoro group and protein, which probable well explained the higher potency of these series inhibitors. More biological evaluation about these gem-difluoromethylenated statin derivatives **10a**—**10c** including cell assay, PK, and small animal models is underway and will be reported in due course.

Acknowledgement

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[10] Check the Sigma-Aldrich's product information of HMG-CoA reductase assay kit for detail.

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