Discovery of DF-461, a Potent Squalene Synthase Inhibitor

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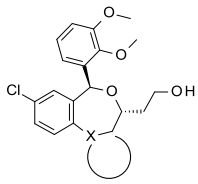
1. Associated content

Although unsubstituted triazole **S2** showed lesser squalene synthase inhibitory activity than basic pyrrole **S1**, chloro and methyl substituted triazole **S3** and **15** demonstrated improved SSI activity (30 nM and 12 nM, respectively).

Table S1. Identification of five-membered hetero aryl rings on new tricyclic template (*trans* racemate)



Compound	Ring	SSI (IC_{50}, nM)
S1		**35**
S2		**>1000**
S3		**30**
15		**12**

SSI : Squalene Synthase Inhibitory activity.

Table S2. Liver selectivity from plasma and liver concentrations in rats (3 mg/kg po)

Compound	t (h)	Live Conc. (ng/g)	Plasma Conc. (ng/ml)	Kp liver
TAK-475	1	703	12	59
22	1	1045	16	66
22	4	569	3	219
25	1	1159	603	19
25	4	623	13	48
26	1	2894	36	80
26	4	868	5	185

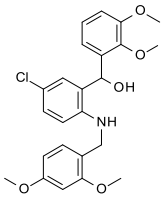
T: time after oral administration. Liver Conc.: liver total drug concentrations (ng/g fresh liver). Plasma Conc.: plasma total drug concentrations (ng/ml).

2. Experimental.

2.1. Chemistry

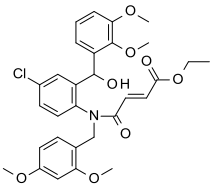
General. Unless otherwise noted, materials were obtained from commercial suppliers and used without further purification. 1H NMR spectra were recorded on JEOL JNM-EX400 spectrometers, and chemical shifts are given in ppm from tetramethylsilane as an internal standard. Parenthetical peak derives from minor atropisomer. FAB mass spectra were recorded on a JEOL JMS-HX110 spectrometer. HR-FAB mass spectra were recorded on a JEOL JMS-700. ESI mass spectra were recorded on SCIEX API-150EX and Agilent Technologies Agilent 1100 series LC/MS. Optical rotations were recorded on a Autopol V plus. Column chromatography was performed with Merck silica gel 60 (particle size 0.060-0.200 or 0.040-0.063). Flash column chromatography was performed with YAMAZEN cartridge series or Ultra Pack series. Thin-layer chromatography (TLC) was performed on Merck pre-coated TLC glass sheets with silica gel 60F254 or Whatman Partisil PLK5F with Silica gel 150Å.

{5-Chloro-2-[(2,4-dimethoxybenzyl)amino]phenyl}-(2,3-dimethoxyphenyl)methanol



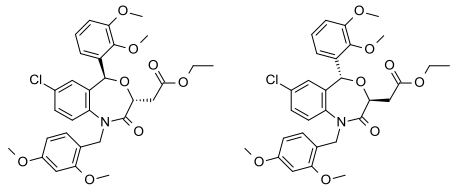
(2-Amino-5-chlorophenyl)(2,3-dimethoxyphenyl)methanol (106 g, 359 mmol) and 2,4-dimethoxybenzaldehyde (62.7 g, 377 mmol) were dissolved in a solvent mixture of methanol (1500 ml) and acetic acid (750 ml). Molecular Sieves 3A powder was added to the resulting solution and the mixture was stirred at 60°C for 2 hours. After the reaction mixture was cooled to room temperature, sodium cyanoborohydride (27.0 g, 430 mmol) was added and the resulting mixture was stirred at room temperature for 3 hours. The reaction mixture was concentrated. The residue was diluted with ethyl acetate and washed with a saturated aqueous solution of sodium bicarbonate and saturated brine. The organic layer was dried over anhydrous sodium sulfate. After concentration of the filtrate, the residue was purified by silica gel column (ethyl acetate : hexane = 1 : 4) to give the title compound (97 g, 61%). MS m/z 444 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 3.77 (3H, s), 3.78 (3H, s), 3.79 (3H, s), 3.88 (3H, s), 4.24 (2H, s), 6.02 (1H, s), 6.38 (1H, dd, J = 8.3, 2.4 Hz), 6.44 (1H, d, J = 2.4 Hz), 6.59 (1H, d, J = 8.8 Hz), 6.84 (1H, dd, J = 7.8, 1.5 Hz), 6.91 (1H, dd, J = 8.1, 1.5 Hz), 6.98-6.99 (1H, m), 7.03-7.08 (3H, m).

Ethyl (E)-4-{4-chloro(2,4-dimethoxybenzyl)-2-[(2,3-dimethoxyphenyl)(hydroxy)methyl]anilino}-4-oxo-2-butenoate



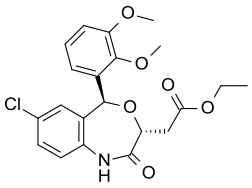
{5-Chloro-2-[(2,4-dimethoxybenzyl)amino]phenyl}(2,3-dimethoxyphenyl)methanol (97 g, 219 mmol) was dissolved in dichloromethane (1000 mL). Sodium hydrogen carbonate (37 g, 438 mmol) and a methylene chloride solution (500 mL) of monoethyl chlorofumarate (53 g, 328 mmol) prepared separately were added dropwise to the resulting solution at 0°C. The mixture was stirred overnight at room temperature. The reaction mixture was diluted with dichloromethane and washed with a saturated aqueous solution of sodium bicarbonate and saturated brine. The organic layer was dried over anhydrous sodium sulfate. The filtrate was then concentrated to give the title compound (137 g, quant.).

Ethyl 2-[(*trans*)-7-chloro-1-(2,4-dimethoxybenzyl)-5-(2,3-dimethoxyphenyl)-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]acetate



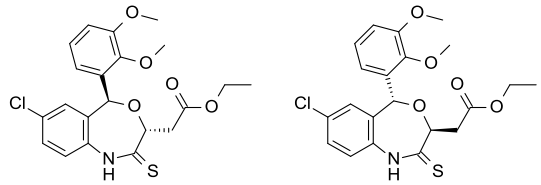
Ethyl (E)-4-{4-chloro(2,4-dimethoxybenzyl)-2-[(2,3-dimethoxyphenyl)(hydroxy)methyl]anilino}-4-oxo-2-butenoate (137 g, 240 mmol) was dissolved in ethanol (1500 mL). Potassium carbonate (50 g, 360 mmol) was added to the solution and the resulting mixture was stirred overnight at room temperature. The reaction mixture was concentrated. The residue was diluted with ethyl acetate and washed with saturated brine. The organic layer was dried over anhydrous sodium sulfate. The filtrate was then concentrated. Crystals thus formed were collected by filtration while using hexane to give the title trans isomer (75 g, 55%). The base solution was concentrated and the residue thus obtained was purified by silica gel column (ethyl acetate : hexane = 1 : 3) to give the cis isomer (24 g, 17%). Trans Isomer MS m/z 570 (M + H) +. 1H-NMR (CDCl3) δ 1.25 (3H, t, J = 7.1 Hz), 2.79 (1H, dd, J = 16.4, 6.1 Hz), 3.09 (1H, dd, J = 16.6, 7.8 Hz), 3.20 (3H, s), 3.66 (3H, s), 3.76 (3H, s), 3.86 (3H, s), 4.13-4.15 (2H, m), 4.48 (1H, dd, J = 7.8, 6.1 Hz), 4.86 (1H, d, J = 14.9 Hz), 5.49 (1H, d, J = 14.9 Hz), 5.97 (1H, s), 6.40-6.42 (2H, m), 6.54 (1H, d, J = 2.2 Hz), 6.92-6.94 (1H, m), 7.13-7.15 (2H, m), 7.23-7.25 (1H, m), 7.29-7.32 (2H,m).

Ethyl 2-[(*trans*)-7-chloro-5-(2,3-dimethoxyphenyl)-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]acetate



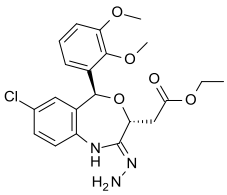
Ethyl 2-[(*trans*)-7-chloro-1-(2,4-dimethoxybenzyl)-5-(2,3-dimethoxyphenyl)-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]acetate (6.00 g, 10.5 mmol) was dissolved in acetone (160 mL). At 0°C, an aqueous solution (40 mL) of ammonium dicerium (IV) nitrate (17.3 g, 31.6 mmol) was added to the solution and the resulting mixture was stirred at room temperature for one hour. A saturated aqueous solution of sodium bicarbonate was added to the reaction mixture, followed by extraction with ethyl acetate. The extract was washed with saturated brine. The organic layer was dried over anhydrous sodium sulfate. The filtrate was then concentrated. The residue was purified by silica gel column (ethyl acetate : hexane = 1 : 2) to give the title compound (3.58 g, 81%). MS m/z 420 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 1.22 (3H, t, J = 7.2 Hz), 2.80 (1H, dd, J = 16.5, 6.7 Hz), 3.04 (1H, dd, J = 16.5, 6.7 Hz), 3.65 (3H, s), 3.89 (3H, s), 4.08-4.13 (2H, m), 4.65 (1H, t, J = 6.7 Hz), 6.22 (1H, s), 6.68 (1H, d, J = 2.4 Hz), 6.97 (1H, dd, J = 8.1, 1.7 Hz), 7.01 (1H, d, J = 8.5 Hz), 7.05-7.08 (1H, m), 7.15 (1H, t, J = 8.1 Hz), 7.27 (1H, dd, J = 8.5, 2.4 Hz).

Ethyl 2-[(*trans*)-7-chloro-1-(2,4-dimethoxybenzyl)-5-(2,3-dimethoxyphenyl)-2-thioxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]acetate



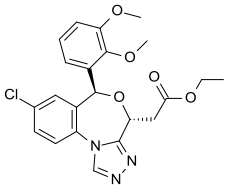
Ethyl 2-[(*trans*)-7-chloro-5-(2,3-dimethoxyphenyl)-2-oxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]acetate (3.00 g, 7.15 mmol) was dissolved in toluene (100 mL). Lawesson's reagent (4.91 g, 12.2 mmol) was added to the solution. The resulting mixture was heated under reflux for one hour. After cooling to room temperature, the reaction mixture was concentrated. The residue was purified twice by silica gel column (chloroform, ethyl acetate : hexane = 1 : 3) to give the cis isomer (0.97 g, 31%) and the title trans isomer (1.27 g, 41%). Trans isomer MS m/z 436 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 1.24 (3H, t, J = 7.1 Hz), 2.96 (1H, dd, J = 16.5, 6.5 Hz), 3.26 (1H, dd, J = 16.5, 6.7 Hz), 3.63 (3H, s), 3.88 (3H, s), 4.10-4.16 (3H, m), 4.71 (1H, t, J = 6.6 Hz), 6.13 (1H, s), 6.66 (1H, d, J = 2.2 Hz), 6.97 (1H, dd, J = 8.1, 2.0 Hz), 7.06 (1H, d, J = 8.1 Hz), 7.10-7.13 (1H, m), 7.16 (1H, t, J = 8.1 Hz), 7.33 (1H, dd, J = 8.1, 2.2 Hz), 9.70 (1H, s).

Ethyl 2-[(*trans*)-7-chloro-5-(2,3-dimethoxyphenyl)-2-[hydrazono]-1,5-dihydro-4,1-benzoxazepin3(3H)-yl]acetate



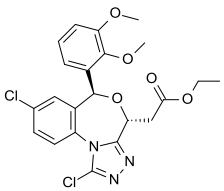
Ethyl 2-[(*trans*)-7-chloro-5-(2,3-dimethoxyphenyl)-2-thioxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]acetate (129 mg, 0.29 mmol) was dissolved in ethanol (3 mL). Hydrazine monohydrate (0.028 mL, 0.53 mmol) was added and the resulting mixture was stirred at room temperature for one hour. The reaction mixture was concentrated to give the title compound (128 mg, quant.). MS (ESI) m/z 434 $(M + H)^+$.

Ethyl 2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate



Ethyl 2-[(*trans*)-7-chloro-5-(2,3-dimethoxyphenyl)-2-[hydrazono]-1,5-dihydro-4,1-benzoxazepin3(3H)-yl]acetate (199 mg, 0.46 mmol) was dissolved in chloroform (6 mL). Ethyl orthoformate (0.382 mL, 2.29 mmol) and sulfuric acid (0.092 mL, 1.72 mmol) were added to the solution. The resulting mixture was stirred at room temperature for 3 hours. The reaction mixture was diluted with chloroform and washed with a saturated aqueous solution of sodium bicarbonate and saturated brine. The organic layer was dried over anhydrous sodium sulfate. After concentration of the filtrate, the residue thus obtained was purified by preparative TLC (methanol : chloroform = 1 : 9) to give the title compound (155 mg, 76%). MS m/z 444 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 1.26 (3H, t, J = 7.2 Hz), 3.22 (1H, dd, J = 16.6, 7.8 Hz), 3.47 (1H, dd, J = 16.5, 5.5 Hz), 3.54 (3H, s), 3.87 (3H, s), 4.15-4.20 (2H, m), 5.16 (1H, dd, J = 7.8, 5.6 Hz), 5.74 (1H, s), 6.80 (1H, d, J = 2.2 Hz), 6.98 (1H, dd, J = 7.6, 2.2 Hz), 7.16-7.23 (2H, m), 7.42 (1H, d, J = 8.5 Hz), 7.47 (1H, dd, J = 8.5, 2.2 Hz), 8.61 (1H, s).

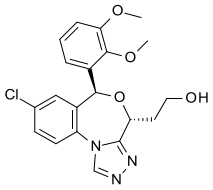
Ethyl 2-[(*trans*)-1,8-dichloro-6-(2,3-dimethoxyphenyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate



Ethyl 2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate (155 mg, 0.35 mmol) was dissolved in carbon tetrachloride

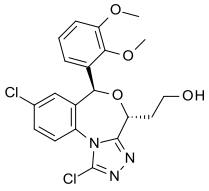
(4 mL). *N*-chlorosuccinimide (75 mg, 0.55 mmol) was added to the solution. The resulting mixture was heated under reflux overnight. The reaction mixture was cooled to room temperature and then concentrated. The residue was diluted with chloroform and washed with a saturated aqueous solution of sodium bicarbonate and saturated brine. The organic layer was dried over anhydrous sodium sulfate. After concentration of the filtrate, the residue thus obtained was purified by silica gel column (methanol : chloroform = 1 : 9) to give the title compound (62 mg, 37%). MS m/z 478 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 1.26-1.30 (3H, m), 3.19 (1H, dd, J = 16.6, 7.3 Hz), 3.43 (1H, dd, J = 16.6, 6.3 Hz), 3.50 (3H, s), 3.87 (3H, s), 4.16-4.22 (2H, m), 4.95 (1H, dd, J = 7.3, 6.3 Hz), 5.66 (1H, s), 6.84 (1H, d, J = 2.2 Hz), 6.98 (1H, dd, J = 7.6, 2.2 Hz), 7.16-7.22 (2H, m), 7.50-7.58 (2H, m).

2-[(*trans*)-8-Chloro-6-(2,3-dimethoxyphenyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]-1-ethanol



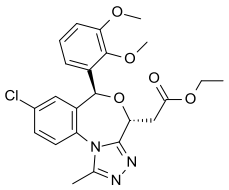
Lithium aluminum hydride (27 mg, 0.20 mmol) was suspended in tetrahydrofuran (1 mL). While being stirred at -15°C, a solution of ethyl 2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate (58 mg, 0.13 mmol) in tetrahydrofuran (1 mL) was slowly added dropwise to the resulting suspension. The reaction mixture was stirred for 15 minutes. An aqueous solution of potassium sodium tartrate was added and the resulting mixture was extracted with chloroform. The organic layer was dried over anhydrous sodium sulfate. The filtrate was concentrated to give the title compound (37 mg, 70%). MS m/z 402 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 2.47-2.64 (2H, m), 3.55 (3H, s), 3.88 (3H, s), 3.91-4.01 (2H, m), 4.87 (1H, dd, J = 7.0, 5.5 Hz), 5.76 (1H, s), 6.82 (1H, d, J = 2.4 Hz), 6.99 (1H, dd, J = 7.3, 2.2 Hz), 7.18-7.25 (2H, m), 7.41-7.49 (2H, m), 8.61 (1H, s).

2-[(*trans*)-1,8-Dichloro-6-(2,3-dimethoxyphenyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]-1-ethanol



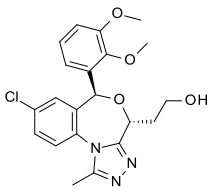
Lithium aluminum hydride (27 mg, 0.20 mmol) was suspended in tetrahydrofuran (1 mL). Under stirring at -15°C, a solution of ethyl 2-[(*trans*)-1,8-dichloro-6-(2,3-dimethoxyphenyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate (62 mg, 0.13 mmol) in tetrahydrofuran (1 mL) was slowly added dropwise to the resulting suspension. The reaction mixture was stirred for 15 minutes. An aqueous solution of potassium sodium tartrate was added and the resulting mixture was extracted with chloroform. The organic layer was dried over anhydrous sodium sulfate. The filtrate was concentrated to give the title compound (33 mg, 58%). MS m/z 436 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 2.48-2.58 (3H, m), 3.51 (3H, s), 3.87 (3H, s), 3.92-3.99 (2H, m), 4.66-4.69 (1H, m), 5.67 (1H, s), 6.85 (1H, d, J = 2.0 Hz), 6.99 (1H, dd, J = 7.8, 1.7 Hz), 7.18-7.25 (2H, m), 7.52-7.57 (2H, m).

Ethyl 2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-methyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]-benzoxazepin-4-yl]acetate



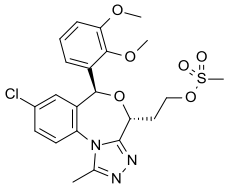
To a solution of ethyl 2-[(*trans*)-7-chloro-5-(2,3-dimethoxyphenyl)-2-thioxo-1,2,3,5-tetrahydro-4,1-benzoxazepin-3-yl]acetate (73.2 mg, 0.168 mmol) in isopropyl alcohol (10 mL) was added acetylhydrazine (37.3 mg, 0.501 mmol). The resulting mixture was heated under reflux. The same amount of acetylhydrazine was added a further three times in total. After confirmation of the disappearance of the raw material, the resulting mixture was concentrated under reduced pressure. After the residue was dissolved in acetic acid (10 mL), the resulting solution was heated under reflux for 75 minutes, followed by concentration under reduced pressure. Chloroform was dissolved in the residue. The resulting solution was washed successively with a saturated aqueous solution of sodium hydrogen carbonate and saturated brine, and dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated under reduced pressure. The crude product was purified by thin layer chromatography (dichloromethane : methanol = 9 : 1) to give the title compound (75.4 mg, 98%). ^{1}H-NMR ($CDCl_3$) δ 1.28 (3H, t, J = 7.1 Hz), 2.66 (3H, s), 3.21 (1H, dd, J = 16.4, 7.6 Hz), 3.41-3.46 (1H, m), 3.49 (3H, d, J = 2.7 Hz), 3.86 (3H, s), 4.18 (2H, dtd, J = 16.8, 6.1, 3.0 Hz), 4.94 (1H, dd, J = 7.7, 6.0 Hz), 5.60 (1H, s), 6.82 (1H, d, J = 2.4 Hz), 6.97 (1H, dd, J = 8.1, 1.7 Hz), 7.20 (2H, tt, J = 11.0, 4.0 Hz), 7.32 (1H, d, J = 8.5 Hz), 7.49 (1H, dd, J = 8.5, 2.4 Hz).

2-[(*trans*)-8-Chloro-6-(2,3-dimethoxyphenyl)-1-methyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]-benzoxazepin-4-yl]-1-ethanol



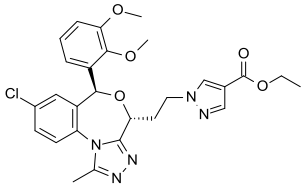
A solution of ethyl 2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-methyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]-benzoxazepin-4-yl]acetate (94.6 mg, 0.207 mmol) in tetrahydrofuran (5.0 mL) was added to a solution of lithium aluminum hydride (11.8 mg, 0.311 mmol) in tetrahydrofuran (1.0 mL)under ice cooling. The resulting mixture was stirred at the same temperature for one hour. To terminate the reaction, a 10% aqueous solution of potassium sodium tartrate was added to the solution. The reaction mixture was extracted three times with chloroform. The organic layers were combined and dried over anhydrous sodium sulfate. After filtration, the filtrate was concentrated under reduced pressure. The residue was purified by thin layer chromatography (dichloromethane : methanol = 9 : 1) to give the title compound (62 mg, 72%). ^{1}H-NMR ($CDCl_3$) δ 2.50-2.56 (2H, m), 2.66 (3H, s), 3.17 (1H, t, J = 5.5 Hz), 3.50 (3H, s), 3.85-4.02 (5H, m), 4.64 (1H, t, J = 6.1 Hz), 5.61 (1H, s), 6.84 (1H, d, J = 2.2 Hz), 6.98 (1H, dd, J = 7.9, 1.6 Hz), 7.18-7.24 (2H, m), 7.32 (1H, d, J = 8.5 Hz), 7.49 (1H, dd, J = 8.4, 2.3 Hz).

2-[(*trans*)-8-Chloro-6-(2,3-dimethoxyphenyl)-1-methyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl methanesulfonate



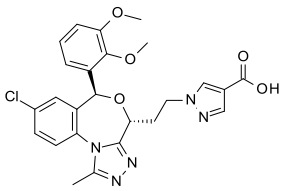
2-[(*trans*)-8-Chloro-6-(2,3-dimethoxyphenyl)-1-methyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]-1-ethanol (57 mg, 0.14 mmol) was dissolved in dichloromethane (2 mL). Triethylamine (0.028 mL, 0.20 mmol) and methanesulfonyl chloride (0.016 mL, 0.20 mmol) were sequentially added to the solution at 0°C, followed by stirring for 0.5 hour. A saturated aqueous solution of sodium bicarbonate was added to the solution, followed by extraction with chloroform. The organic layer was dried over anhydrous sodium sulfate to give the title compound (89 mg, quant.).

Ethyl 1-{2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-methyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl-1H-pyrazole-4-carboxylate



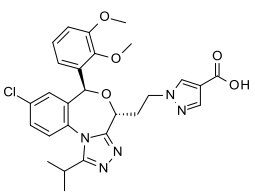
Ethyl 4-pyrazolecarboxylate (52 mg, 0.37 mmol) was dissolved in *N*,*N*-dimethylformamide (1 mL). At 0°C, sodium hydride (55%, 16 mg, 0.37 mmol) was added to the solution and the resulting mixture was stirred for 0.5 hour at 0°C. A solution of 2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-methyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl methanesulfonate (121 mg, 0.25 mmol) in *N*,*N*-dimethylformamide (2 mL) was added dropwise to the reaction mixture. Tetrabutylammonium iodide (91 mg, 0.25 mmol) was further added. The resulting mixture was stirred overnight at 50°C. Water was added to the reaction mixture and the resulting mixture was extracted with chloroform. The organic layer was dried over anhydrous magnesium sulfate. The filtrate was then concentrated. The residue thus obtained was purified by preparative TLC (methanol : chloroform = 1 : 9) to give the title compound (78 mg, 59%). MS (ESI) m/z 538 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 1.32 (3H, t, J = 7.1 Hz), 2.66 (3H, s), 2.79-2.87 (2H, m), 3.49 (3H, s), 3.87 (3H, s), 4.26 (2H, q, J = 7.1 Hz), 4.38-4.42 (1H, m), 4.47-4.59 (2H, m), 5.59 (1H, s), 6.82 (1H, d, J = 2.2 Hz), 6.99 (1H, dd, J = 7.6, 2.2 Hz), 7.19-7.23 (2H, m), 7.30 (1H, d, J = 8.5 Hz), 7.48 (1H, dd, J = 8.5, 2.4 Hz), 7.84 (1H, d, J = 0.5 Hz), 7.91 (1H, d, J = 0.5 Hz).

1-{2-[(*trans*)-8-Chloro-6-(2,3-dimethoxyphenyl)-1-methyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl-1H-pyrazole-4-carboxylic acid (4)



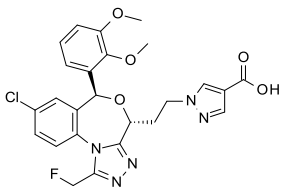
Ethyl 1-{2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-methyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl-1H-pyrazole-4-carboxylate (78 mg, 0.15 mmol) was dissolved in methanol-water-tetrahydrofuran (2 : 1 : 2, 5 ml). Potassium carbonate (60 mg, 0.44 mmol) was added to the resulting solution, followed by stirring at room temperature for 5 days and then at 55°C overnight. After the reaction mixture was neutralized with an acidic resin (Amberlite IR-120B), the resin was filtered out. The residue was washed with methanol. The filtrate was then concentrated. The residue thus obtained was purified by preparative TLC (methanol : chloroform = 1 : 9) to give the title compound (41 mg, 55%). MS (ESI) m/z 510 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 2.62 (3H, s), 2.71-2.83 (2H, m), 3.46 (3H, s), 3.85 (3H, s), 4.36-4.45 (3H, m), 5.56 (1H, s), 6.79 (1H, d, J = 2.2 Hz), 6.96 (1H, dd, J = 8.1, 1.2 Hz), 7.18 (1H, t, J = 7.9 Hz), 7.23 (1H, d, J = 7.3 Hz), 7.30 (1H, d, J = 8.1 Hz), 7.44 (1H, dd, J = 8.1, 2.2 Hz), 7.77 (1H, s), 7.94 (1H, s).

1-{2-[(*trans*)-8-Chloro-6-(2,3-dimethoxyphenyl)-1-isopropyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl-1H-pyrazole-4-carboxylic acid (5)



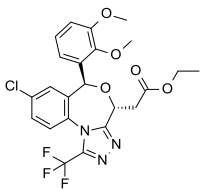
Ethyl 1-{2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-isopropyl-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl-1H-pyrazole-4-carboxylate (70 mg, 0.12 mmol) was dissolved in methanol-water-tetrahydrofuran (1 : 0.5 : 1, 2.5 mL). Lithium hydroxide monohydrate (10 mg, 0.25 mmol) was added to the solution. The resulting mixture was stirred at 50°C for 2 hours. After the reaction mixture was neutralized with an acidic resin (Amberlite IR-120B), the resin was filtered out and the residue was washed with methanol. The filtrate was concentrated and the residue thus obtained was purified by preparative TLC (methanol : chloroform = 1 : 9) to give the title compound (59 mg, 88%). MS (ESI) m/z 538(M + H) $^+$. ^{1}H-NMR ($CDCl_3$) δ 1.13 (3H, d, J = 6.8 Hz), 1.63 (3H, d, J = 6.8 Hz), 2.76-2.88 (2H, m), 3.33-3.40 (1H, m), 3.46 (3H, s), 3.85 (3H, s), 4.37 (1H, t, J = 6.2 Hz), 4.44-4.55 (2H, m), 5.50 (1H, s), 6.78 (1H, d, J = 2.2 Hz), 6.97 (1H, dd, J = 8.1, 1.5 Hz), 7.17-7.24 (2H, m), 7.37 (1H, d, J = 8.3 Hz), 7.45 (1H, dd, J = 8.3, 2.2 Hz), 7.82 (1H, s), 7.96 (1H, s).

1-{2-[(*trans*)-8-Chloro-6-(2,3-dimethoxyphenyl)-1-(fluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl-1H-pyrazole-4-carboxylic acid (6)



Ethyl 1-{2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-(fluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl-1H-pyrazole-4-carboxylate (19 mg, 0.03 mmol) was dissolved in methanol-water-tetrahydrofuran (1 : 0.5 : 1, 2.5 mL). Lithium hydroxide monohydrate (4.3 mg, 0.10 mmol) was added to the solution. At 50°C, the resulting mixture was stirred overnight. After the reaction mixture was neutralized with an acidic resin (Amberlite IR-120B), the resin was filtered out and the residue was washed with methanol. The filtrate was concentrated and the residue thus obtained was purified by preparative TLC (methanol : chloroform = 1 : 9) to give the title compound (13 mg, 72%). MS (ESI) m/z 528 (M + H) $^+$. ^{1}H-NMR ($CDCl_3$) δ 2.72-2.84 (2H, m), 3.38 (3H, s), 3.79 (3H, s), 4.40-4.46 (3H, m), 5.34 (1H, dd, J = 48.3, 11.7 Hz), 5.49 (1H, s), 5.78 (1H, dd, J = 48.3, 11.7 Hz), 6.76 (1H, d, J = 2.2 Hz), 6.91 (1H, dd, J = 7.6, 2.0 Hz), 7.11-7.17 (2H, m), 7.41-7.45 (1H, m), 7.52 (1H, d, J = 8.3 Hz), 7.77 (1H, s), 7.88 (1H, s).

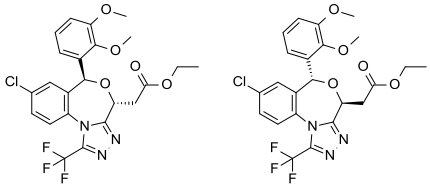
Ethyl 2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate



Ethyl 2-[(*trans*)-7-chloro-5-(2,3-dimethoxyphenyl)-2-[hydrazono]-1,5-dihydro-4,1-benzoxazepin-(3H)-yl]acetate (30 mg, 0.07 mmol) was dissolved in dichloromethane (3 mL). Trifluoroacetic anhydride (0.027 mL, 0.19 mmol) and trifluoroacetic acid (0.106 mL, 1.38 mmol) was added to the solution. The resulting mixture was stirred at room temperature for one hour. After stirring the reaction mixture at 55°C and distilling off dichloromethane, toluene (3 mL) was added to the residue. The resulting mixture was heated under reflux for a further one hour. After cooling to room temperature, the reaction mixture was concentrated. The residue was diluted with dichloromethane, and washed with a saturated aqueous solution of sodium bicarbonate. The organic layer was dried over anhydrous sodium sulfate. After concentration of the filtrate, the residue thus obtained was purified by preparative TLC (methanol : dichloromethane = 1 : 9) to give the title compound (24 mg, 69%). MS (ESI) m/z 512 (M + H) $^+$. ^{1}H-NMR ($CDCl_3$) δ 1.28 (3H, td, J = 7.1, 1.4 Hz), 3.24 (1H, dd, J = 16.7, 7.0 Hz), 3.43 (3H, d, J = 1.2 Hz), 3.49 (1H, dd, J = 16.7, 6.5 Hz), 3.86 (3H, d, J = 1.2 Hz), 4.19 (2H, ddd, J = 14.3, 7.1, 1.5 Hz), 4.93 (1H, t, J = 6.8 Hz), 5.55 (1H, s), 6.85 (1H, d, J = 1.2 Hz) ,6.98 (1H, dd, J = 6.8, 2.9 Hz), 7.18 (2H, dd, J = 9.5, 5.1 Hz), 7.52 (2H, s).

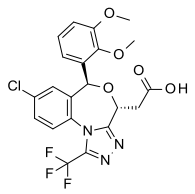
Ethyl 2-[(4*R*,6*S*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]-triazolo-[4,3-a][4,1]benzoxazepin-4-yl]acetate (isomer A)

Ethyl 2-[(4*S*,6*R*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]-triazolo-[4,3-a][4,1]benzoxazepin-4-yl]acetate (isomer B)



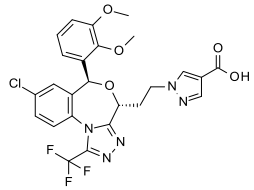
The racemic compound (1138 mg) was optically resolved by an optically active column (CHIRALPAK-AD; 5 cm × 50 cm) into isomer A (496 mg) and isomer B (500 mg). Isomer A was an isomer with a short retention time, while isomer B was an isomer with a long retention time. Flow rate: 50 mL/min. Developing solvent: 15% ethanol-*n*-hexane.

2-[(4*R*,6*S*)-8-Chloro-6-(2,3-dimethoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]-triazolo-[4,3-a][4,1]benzoxazepin-4-yl]acetic acid (19)



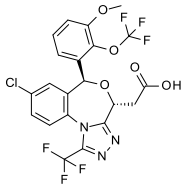
Ethyl 2-[(4*R*,6*S*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]-triazolo-[4,3-a][4,1]benzoxazepin-4-yl]acetate (500 mg, 0.98 mmol) was dissolved in 1,4-dioxane (15 mL). Concentrated hydrochloric acid (3.3 mL) was added to the solution. The resulting mixture was stirred at 60°C for 21 hours. After cooling to room temperature, the reaction mixture was diluted with ethyl acetate and washed with water. The organic layer was dried over anhydrous sodium sulfate. The filtrate was then concentrated. The residue obtained by concentration was purified by preparative TLC (methanol : chloroform = 1 : 9) to give the title compound (333 mg, 70%). $[\alpha]_D^{20}$ -46.2° (C = 0.50, MeOH). MS (ESI) m/z 484 (M + H)$^+$. ^{1}H-NMR ($CDCl_3$) δ 3.01-3.04 (1H, m), 3.25-3.27 (1H, m), 3.40 (3H, s), 3.84 (3H, s), 4.84-4.86 (1H, m), 5.49 (1H, s), 6.80 (1H, d, J = 2.2 Hz), 6.93 (1H, d, J = 7.9 Hz), 7.13 (1H, t, J = 7.9 Hz), 7.21 (1H, d, J = 7.9 Hz), 7.39 (1H, dd, J = 8.5, 2.2 Hz), 7.49 (1H, d, J = 8.5 Hz).

1-{2-[(*trans*)-8-Chloro-6-(2,3-dimethoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl-1H-pyrazole-4-carboxylic acid (7)



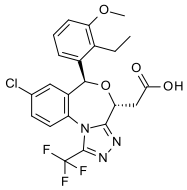
Ethyl 1-{2-[(*trans*)-8-chloro-6-(2,3-dimethoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]ethyl-1H-pyrazole-4-carboxylate (136 mg, 0.23 mmol) was dissolved in ethanol-water-tetrahydrofuran (10 : 5 : 5, 20 mL). Lithium hydroxide monohydrate (19 mg, 0.46 mmol) was added to the solution and the resulting mixture was stirred overnight at 50°C. The reaction mixture was neutralized with 1N hydrochloric acid and then, extracted with dichloromethane. The organic layer was dried over anhydrous sodium sulfate. After concentration of the filtrate, the residue thus obtained was purified by preparative TLC (methanol : dichloromethane = 1 : 9) to give the title compound (120 mg, 93%). MS (ESI) m/z 564 (M + H)$^+$. ^{1}H-NMR ($CDCl_3$) δ 2.87-2.93 (2H, m), 3.43 (3H, s), 3.86 (3H, s), 4.41 (1H, t, J = 6.3 Hz), 4.56 (2H, dt, J = 18.8, 6.9 Hz), 5.55 (1H, s), 6.85 (1H, d, J = 1.7 Hz), 7.00 (1H, dd, J = 6.2, 3.5 Hz), 7.21 (2H, t, J = 3.1 Hz), 7.50 (2H, dt, J = 10.7, 4.5 Hz), 7.88 (1H, s), 7.97 (1H, s). IR(ATR)cm^{-1} 1712, 1685, 1556, 1483, 1284, 1200, 1171, 1146, 1074, 995, 768. HRMS (FAB) m/z Calculated: $C_{25}H_{22}O_5N_5ClF_3$; 564.1262. Found: 564.1245.

2-[(4*R*,6*S*)-8-Chloro-6-[3-methoxy-2-(trifluoromethoxy)phenyl]-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetic acid (20)



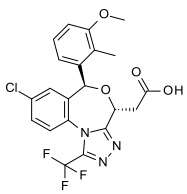
In a similar manner to that employed for the synthesis of compound **19**, the title compound (1.11 g, 87%) was obtained from ethyl 2-[(4*R*,6*S*)-8-chloro-6-[3-methoxy-2-(trifluoromethoxy)phenyl]-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate (1.18 g, 2.09 mmol). $[\alpha]_D^{20}$ -17.2° (C = 0.50, MeOH). MS (FAB) m/z 538 (M + H)$^+$. ^{1}H-NMR ($CDCl_3$) δ 3.30 (1H, dd, J = 17.2, 6.8 Hz), 3.57 (1H, dd, J = 17.2, 6.8 Hz), 3.88 (3H, s), 4.91 (1H, t, J = 6.8 Hz), 5.55 (1H, s), 6.84 (1H, d, J = 2.0 Hz), 7.07 (1H, dd, J = 8.3, 1.5 Hz), 7.31 (1H, dd, J = 7.8, 1.0 Hz), 7.42 (1H, t, J = 8.3 Hz), 7.55 (2H, t, J = 2.8 Hz).

[(4*R*,6*S*)-8-Chloro-6-(2-ethyl-3-methoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetic acid (21)



In a similar manner to that employed for the synthesis of compound **19**, the title compound (181 mg, 83%) was obtained from ethyl [(4*R*,6*S*)-8-chloro-6-(2-ethyl-3-methoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate (232 mg, 0.45 mmol). $[\alpha]_D^{20}$ -29.3° (C = 0.51, MeOH). MS (ESI) m/z 482 (M + H)$^+$. ^{1}H-NMR ($CDCl_3$) δ 0.71 (3H, t, J = 7.5 Hz), 2.00-2.11 (1H, m), 2.20-2.32 (1H, m), 3.32 (1H, dd, J = 17.2, 6.7 Hz), 3.58 (1H, dd, J = 17.2, 6.7 Hz), 3.84 (3H, s), 4.93 (1H, t, J = 6.7 Hz), 5.43 (1H, s), 6.86 (1H, s), 6.92 (1H, d, J = 8.3 Hz), 7.25 (1H, d, J = 7.8 Hz), 7.32 (1H, t, J = 7.8 Hz), 7.51-7.57 (2H, m).

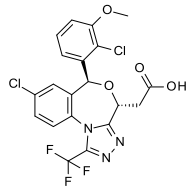
2-[(4*R*,6*S*)-8-Chloro-6-(3-methoxy-2-methylphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetic acid (22)



In a similar manner to that employed for the synthesis of compound **19**, the title compound (140 mg, 95%) was obtained from ethyl 2-[(4*R*,6*S*)-8-chloro-6-(3-methoxy-2-methylphenyl)-1-(trifluoromethyl)-4H,6H-

[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate(157 mg, 0.32 mmol) by using concentrated hydrochloric acid (3.0 mL). $[\alpha]_D^{20}$ -20.6° (C = 0.50, MeOH). MS (ESI) m/z 468 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 1.61 (3H, s), 3.15-3.27 (1H, m), 3.35-3.52 (1H, m), 3.81 (3H, s), 4.85-4.93 (1H, m), 5.29-5.33 (1H, m), 6.74-6.77 (1H, m), 6.84-6.90 (1H, m), 7.24-7.28 (2H, m), 7.54-7.45 (2H, m).

2-[(4*R*,6*S*)-8-Chloro-6-(2-chloro-3-methoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetic acid (23)



In a similar manner to that employed for the synthesis of compound **19**, the title compound (131 mg, 53%) was obtained from ethyl 2-[(4*R*,6*S*)-8-chloro-6-(2-chloro-3-methoxyphenyl)-1-(trifluoromethyl)-4H,6H-[1,2,4]triazolo[4,3-a][4,1]benzoxazepin-4-yl]acetate (261 mg, 0.51 mmol) by using concentrated hydrochloric acid (1.1 mL). $[\alpha]_D^{20}$ -28.1° (C = 0.50, MeOH). MS (ESI) m/z 488 $(M + H)^+$. ^{1}H-NMR ($CDCl_3$) δ 3.19-3.23 (1H, m), 3.41-3.43 (1H, m), 3.89 (3H, s), 4.89 (1H, t, J = 6.7 Hz), 5.47 (1H, s), 6.69-6.70 (1H, m), 6.96-6.98 (1H, m), 7.35-7.37 (2H, m), 7.51-7.52 (2H, m).

2.2. Biological evaluation procedure of inhibitory effects on cholesterol synthesis in rat hepatic cells.

SSI IC_{50} values were measured using by slightly modified Shechter's method[23]. And CSI IC_{50} values were measured as described below.

2.2.1. Preparation of rat primary hepatocytes.

Shechter's method[23] was slightly modified. This study consisted of three experiments, and the effects of inhibitors at each concentration were evaluated in triplicate. One animal was used to prepare the hepatocytes for each experiment. Under anesthesia by thiopental sodium (0.1 g/kg, i.p.), a plastic catheter was introduced through the portal vein. The liver was perfused with Ca^{2+}, Mg^{2+} free Hanks' balanced salts solution (pH 7.2) containing 2% albumin, 0.5 mM EGTA, 10 mM HEPES, and 41.7 mM $NaHCO_3$ at 37°C for 10 min at 19 - 21 mL/min; and then with Ca^{2+}, Mg^{2+} free Hanks' balanced salts solution (pH 7.5) containing 0.05% collagenase, 4 mM $CaCl_2$, 10 mM HEPES, and 41.7 mM $NaHCO_3$ for another 15 minutes. Liver cells were dispersed in DMEM supplemented with 100 U/mL penicillin and 100 μg/ml streptomycin by dissection and gentle pipetting. After filtration through a 70 μm nylon mesh filter (Cell Strainer, BD Falcon), hepatocytes were obtained by repeated centrifugation (3 times) at 600 rpm (centrifuge; 5930, swinging bucket rotor: RS-3011M) for 1 minute at 4°C. After the last centrifugation, the medium was changed to DMEM supplemented with 10% LPDS, 100 U/ml penicillin, and 100 μg/ml streptomycin. Then, viability was determined by staining with trypan blue. Hepatocytes with over 80% viability were cultured in 6-well cell culture plates (10^6 cells/well).

2.2.2. Measurement of cholesterol biosynthetic activity of rat hepatocytes.

The following day, the medium was replaced with media supplemented with 5% LPDS, 25 mM HEPES, and inhibitors (final concentrations: 0, 1, 3, 10, 30, 100, 300, 1000, and 3000 nM). After incubation for 1 hour at 37°C, 10 μl of [^{14}C]mevalonolactone (5 μCi/ml) was added into the media, and the incubation was continued for another 1 hour. The cells were washed with D-PBS (3 times) and dissolved in 1 ml of 0.1 M NaOH. Ten micro liters of the cell lysates were transferred to a 96-well plate to determine the protein concentration in duplicate. Eight hundred microliters of the remains were saponified for 1 hour at 75°C by adding 2 mL of ethanol and 0.5 mL of 50 (w/v)% KOH. After the addition of 50 or 100 μl of [^{3}H]cholesterol (0.45 μCi/ml) as an internal standard, the nonsaponifiable lipids were extracted with 4.5 mL of petroleum ether. The water layer was frozen in dry ice and ethanol, and the upper layer was transferred to another tube. The extracts in the tubes were dried under N_2 gas at 40°C. The residue was dissolved in 50 μl of dichloromethane - methanol (2 : 1) solution including 10 mg/ml cholesterol, applied onto TLC plastic sheets (Silica gel 60), and developed with a solvent (toluene - ethyl acetate, 3 : 1). The radio activities incorporated into the cholesterol fractions in Aquasol-2 were counted with a liquid scintillation counter.

The protein concentration was determined using a BCA Protein Assay Kit.

The radioactivities incorporated into the cholesterol fractions were corrected from the formula as follows:

Radioactivities incorporated (dpm/μg protein) = Radioactivities of [^{14}C]cholesterol (dpm) × 50,000 (dpm) / radioactivities of [^{3}H]cholesterol (dpm) / protein content (μg)

Referring to the mean radioactivity of the cells in the three wells treated with 0 nM of inhibitors, inhibition (%) of cholesterol synthesis at each concentration was calculated by the following equation:

Inhibition (%) = (1 – arithmetic mean radioactivity incorporated of three wells at each concentration / arithmetic mean radioactivity incorporated of three wells at 0 nM) × 100

2.3. Rat single-dose *in vivo* hepatic cholesterol synthesis inhibitory activity.

A rat single-dose liver-cholesterol synthesis inhibitory effect was measured as described below.

To each the compounds was added a necessary amount of a 0.5% methyl cellulose solution immediately before use. Then, an equivalent molar amount of sodium hydroxide or sodium hydrogen carbonate was added to dissolve or suspend it in the resulting solution. 6-week-old

Wistar male rats were orally administered each of the compounds (1 or 3 mg/kg, n = 4-6), while only a 0.5% methyl cellulose solution was administered to the control group. 1,4 or 7 h later, physiological saline of mevalonic acid (5 μCi / 5 ml/kg) labeled with a radioisotope ^{14}C was intraperitoneally administered. The rats were sacrificed 1 hour later. To 1 g of the liver obtained from the rats was added 5 ml of a 15% KOH ethanol solution and the resulting mixture was left to stand for 15 hours. After heating at 75°C for 2 hours, the reaction mixture was extracted with 5 ml of water and 10 ml of petroleum ether. The petroleum ether layer was collected, evaporated to dryness and then dissolved in 50 μl of a chloroform : acetone = 2 : 1 solution. The cholesterol band was separated by silica gel thin layer chromatography (Art.5748, toluene : ethyl acetate = 3 : 1) and cut out. It was placed into a vial container, followed by the addition of 10 ml of Aquasol-2 (product of Packard BioScience Company). The radioactivity was measured using a liquid scintillation counter. A ratio of the radioactivity relative to that of a control group was determined and liver-cholesterol synthesis inhibitory activity (%) was calculated.

A rat ED_{50} values were measured as described below.

DF-461 (0.03, 0.1, and 0.3 mg/kg) or vehicle were orally given to Wistar rats without fasting. 1 hour later, [2-^{14}C]-mevalonolactone (5 μCi/5 mL/kg) was intraperitoneally injected. By using above mentioned method, liver-cholesterol synthesis inhibitory activities (%) were calculated. Radioactivities incorporated in the group treated with vehicle were 24458.96 ± 1862.43 dpm/g tissue. The radioactivities incorporated in the groups treated with **DF-461** were 18045.57 ± 1250.05 dpm/g tissue (0.03 mg/kg), 15267.76 ± 714.93 dpm/g tissue (0.1 mg/kg) and 6122.34 ± 640.64 dpm/g tissue (0.3 mg/kg), respectively.

DF-461 lowered the radioactivities incorporated by 26.22 ± 5.11% (0.03 mg/kg), 37.58 ± 2.92% (0.1 mg/kg) and 74.97 ±2.62% (0.3 mg/kg), respectively. The inhibition was in a dose dependent manner and showed statistical significance from doses of 0.03 mg/kg. ED_{50} and the corresponding 95% confidence interval (CI) were 0.12 (95%CI: 0.09 to 0.16) mg/kg (**Figure S1**).

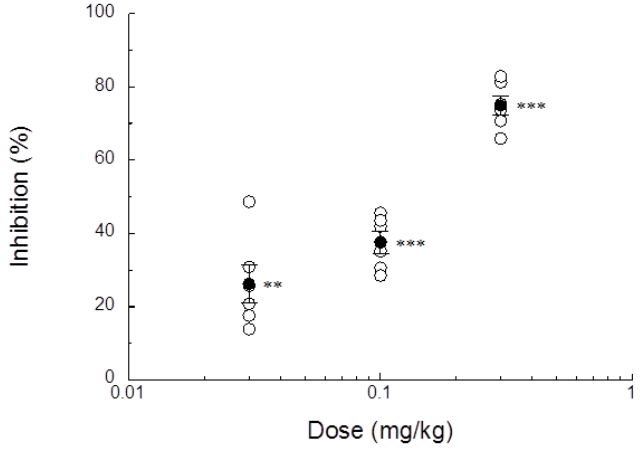


Figure S1 Effects of DF-461 on hepatic cholesterol biosynthesis in Wistar rats.

DF-461 was orally given to Wistar rats. ED_{50} was 0.12 mg/kg (95% CI 0.09 to 0.16 mg/kg). **P<0.01, ***P<0.001 vs vehicle-treated group (Dunnett multiple comparison test). Open circle, individual value; closed circle, mean ± SE of 6 animals.

2.4. Plasma lipid lowering studies in common marmosets.

Male and female common marmosets (305 - 410 g) were purchased from Clea Japan (Tokyo, Japan), fed a commercial chow diet (CMS-1M; Clea Japan) and allowed access to water ad libitum. All animal experiments were carried out according to the Daiichi Sankyo Animal Care Guidelines. Before the experiment, blood samples were collected under nonfasted conditions. Plasma total cholesterol and triglyceride were measured as described above. High-density lipoprotein (HDL) was separated by precipitation reagents (Wako Pure Chemical Industries), and then the cholesterol was measured enzymatically. Non HDL-cholesterol was calculated by subtracting HDL-cholesterol from total cholesterol. Common marmosets were divided into two groups (control vs prepared compound, 30 mg/kg, n = 8); groups were matched for body weight, plasma total cholesterol, triglyceride, HDL cholesterol and non-HDL cholesterol. Drugs were suspended in 0.5% methylcellulose solution and administered orally at 5 ml/kg once a day (9-10AM) for 7 days. The next morning after the final administration of drugs, blood samples were collected under nonfasted conditions and plasma parameters were measured.