Synthesis of FTY720 (Fingolimod) Derivatives Containing Serine Structure

Yoon Sin Oh,[†,‡‡] Taeho Lee,[‡,‡‡] Sang Mi Shin,[§] Jitendra Shrestha,[¶] Doohyun Lee,[‡] Eun-Young Park,[¶,*] and Dong Jae Baek[¶,*]

[†]*Department of Food and Nutrition, Eulji University, Seongnam 13135, South Korea*
[‡]*College of Pharmacy, Research Institute of Pharmaceutical Sciences, Kyungpook National University, Daegu 41566, South Korea*
[§]*College of Pharmacy, Chosun University, Gwangju 61452, South Korea*
[¶]*College of Pharmacy and Natural Medicine Research Institute, Mokpo National University, Jeonnam, 58554, South Korea. *E-mail: parkey@mokpo.ac.kr; dbaek@mokpo.ac.kr*
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FTY720 (Fingolimod, Gilenya) is a fungal metabolite derived from the Chinese herb *Iscaria sinclarii.* It is structurally and biologically similar to sphingosine. FTY720 is phosphorylated (FTY720-P) by sphingosine kinase 2 (SK2) and shows immunosuppressive effects. FTY720-P binds to sphingosine-1-phosphate (S1P) on the lymphocyte surface for decomposition, isolation of lymphocytes from lymph nodes and prevents their release into blood.[1] The mechanism has an immunosuppressive effect in patients with multiple sclerosis.[2] Multiple sclerosis is a major neurological disorder affecting 2.3 million patients worldwide, and occurs mainly in young women.[3] FTY720 is an oral treatment for multiple sclerosis first approved by the US FDA in 2010. In addition, FTY720 activates protein phosphatase 2A (PP2A), which plays a tumor suppressor role, resulting in anticancer effects in cancers of blood, liver, bladder, colon and rectum showing reduced cellular PP2A activity.[4–7] FTY720 stimulates beta cell regeneration in mice and normalizes hyperglycemia.[8,9] Biologically active lipids, such as sphingolipids have been studied for a long time in various disease models. In this study, we investigated the role of FTY720, which is structurally and biologically similar to sphingolipid. A definitive therapeutic agent is unavailable for multiple sclerosis (MS) underscoring the need for discovery and development of selective compounds and derivatives with therapeutic activity. FTY720 carries a polar head group, an aromatic backbone, and an eight-carbon chain. Analysis of the structure–activity relationship of FTY720 derivatives synthesized and reported thus far revealed significant activity associated with the head group.[10,11] Structurally, however, the two hydroxyl groups of FTY720 and the linked *tert*-amine prevent synthesis of various derivatives.[12–14] The polar head group developed so far shows unique physiological activities that differ between FTY720 and modified FTY720 derivatives in stereoselectivity. For example, ROME ((*R*)-FTY720 methyl ether)[11] is known to selectively inhibit SK2. However, FTY720 derivatives in the chiral head group are difficult to synthesize and involve multiple synthetic steps. FTY720 derivatives incorporating serine in the head group yield chiral products in simple synthetic steps. As shown in Scheme 2, compounds **1** and **2**, which are FTY720 derivatives with a known serine structure, show inhibitory effects with IC_{50} values of 5.0 and 4.3 μM for SK1, respectively.[15] Serine-FTY720 derivatives exhibiting such SK1 inhibitory effects display potential anticancer properties. Thus, this study introduced serine, which is commercially available at a low price, to synthesize additional derivatives for anticancer research (Figure 1).

By introducing serine into aniline, we obtained compounds **1** and **2** from compounds **19** and **20**. We obtained compounds **3** and **4** by using Sonogashira coupling to introduce 1-octyne into commercially available 4-iodobenzylamine. The purified product **9** was reduced via hydrogenation using palladium to obtain compound **10** and the method was used to obtain compounds **3** and **4** via EDCI coupling and Boc deprotection. The known compounds **11** and **12** were synthesized[15–17] and mesyl and azide were introduced without purification to obtain compounds **5**–**8**. Similarly, the compounds **15** and **16** so obtained were hydrogenated and reduced to amines. The compounds **17** and **18** were used to obtain the final FTY720 derivatives **5**–**8** similarly (Schemes 1 and 2).

In order to determine the anticancer properties of the newly synthesized sphingosine-serine hybrid compound, we analyzed the degree of cell death in HT29 cells (human colorectal adenocarcinoma cell). Significant cell death was observed with all compounds when tested at a concentration of 40 μM. The most lethal effects were observed in compound **8** (Figure 2(a)). In addition, compound **4** carrying L-serine showed weak anticancer properties (Figure 2(a)). To determine whether the cell death induced by FTY720

[‡‡]These authors contributed equally to this work.

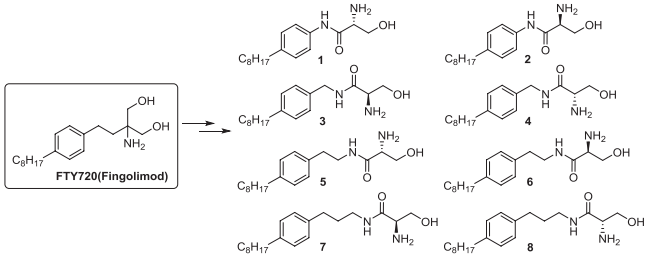
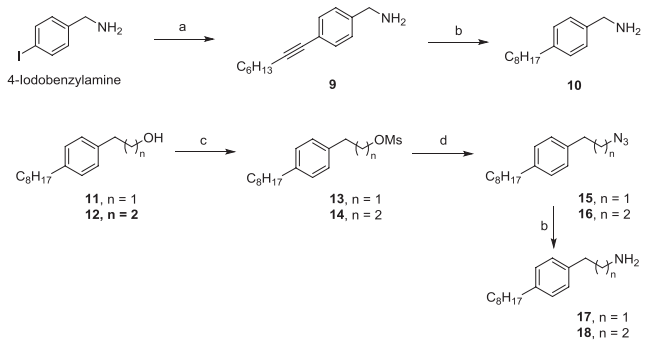
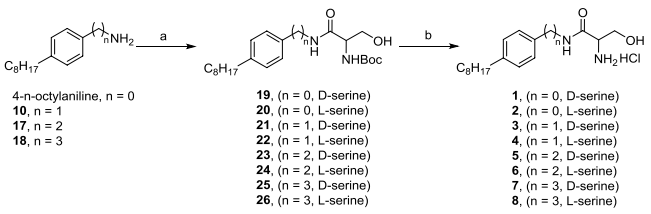


Figure 1. Synthesized FTY720 derivatives.



Scheme 1. Synthesis of compounds **10**, **17**, and **18**. Reagents and conditions: (a) 1-octyne, $Pd(PPh_3)_4$, CuI, NEt_3, 60 °C, 12 h; (b) 5% Pd/C, H_2, EtOAc, rt., 12 h; (c) MsCl, NEt_3, CH_2Cl_2, rt., 6 h; (d) NaN_3, DMF, 80 °C, 12 h.



Scheme 2. Synthesis of compounds **1**–**8**. Reagents and conditions: (a) Boc-D-serine or Boc-L-serine, EDCI, DMAP, CH_2Cl_2, rt., 12 h; (b) 1 M HCl, THF, rt., 12 h.

derivatives was a result of increased apoptosis, we used annexin-V staining. The number of annexin-V stained cells was significantly increased in HT29 cells treated with compound **8** for 24 h (Figure 2(b)). This result showed that the L and D forms of serine exhibit distinct anticancer properties. Our findings highlight the relationship of carbon numbers in serine and phenyl groups with anticancer properties.

Experimental

Synthesis in General. All chemicals were reagent grade and used as purchased. Reactions were run under nitrogen and were monitored by TLC using silica gel 60 F254 aluminum-backed plates. Flash column chromatography was performed on silica gel grade 60 (230–400 mesh). All solvents were of anhydrous quality and were used as received. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker Avance I spectrometer (Billerica, MA, USA), and

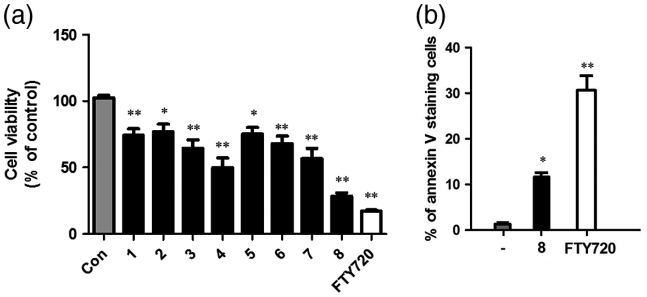


Figure 2. Effects of FTY720 derivatives on HT29 cells viability. (a) HT29 cells were treated with compounds **1**–**8** at 40 μM for 24 h (FTY720 20 μM), and cell viability was measured by EZ-CYTOX Kit (n = 11). (b) Cells were stained with annexin-V-FITC. Apoptotic cells were counted by cytometer. *p < 0.05, **p < 0.01 compared with control cells.

chemical shifts are reported in δ units relative to deuterated solvents, which served as internal references, at 400 and 100 MHz, respectively. High resolution mass spectra were recorded on an Agilent Technologies G6520A Q-TOF mass spectrometer (Santa Clara, CA, USA) using electrospray ionization (ESI). All compounds were ≥95% pure as determined by examining their HRMS and ^{1}H NMR spectra.

(*R*)-2-Amino-3-Hydroxy-*N*-(4-Octylphenyl)Propanamide Hydrochloride 1. To a solution of **19** (100 mg, 0.25 mmol) in THF (5 mL) at 0 °C was added 1 M HCl (1 mL). After being stirred at room temperature for 12 h, the reaction mixture was evaporated and was washed with EtOAc/hexane (1/1), evaporated, and dried. Compound **1** (77 mg, 92%) was obtained as a white solid. ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 10.34 (s, NH), 8.43 (s, NH), 7.59 (d, J = 8.2 Hz, 2H), 7.22 (d, J = 8.2 Hz, 2H), 4.30 (d, J = 4.1 Hz, 1H), 4.19 (dd, J = 12.0, 3.9 Hz, 1H), 4.12 (s, OH), 4.03 (dd, J = 11.8, 6.7 Hz, 1H), 2.66 (t, J = 7.6 Hz, 2H), 1.66 (d, J = 6.2 Hz, 2H), 1.49–1.28 (m, 10H), 0.97 (t, J = 6.3 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 166.6, 141.3, 136.8, 130.5, 121.9, 62.5, 57.3, 37.0, 33.6, 33.2, 31.1, 30.9, 24.3, 15.4; ESI-HRMS $(M + H)^+$ *m/z* calcd for $C_{17}H_{29}N_2O_2$ 293.2229, found 293.2218.

(*S*)-2-Amino-3-Hydroxy-*N*-(4-Octylphenyl)Propanamide Hydrochloride 2. Compound **2** was prepared from **20** according to the same reaction procedure to that described for **1**. Yield = 94%; ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 10.20 (s, NH), 8.38 (s, NH), 7.46 (d, J = 8.4 Hz, 2H), 7.10 (d, J = 8.4 Hz, 2H), 4.11 (dt, J = 7.1, 3.5 Hz, 1H), 4.03 (dd, J = 11.9, 4.2 Hz, 1H), 3.88 (dd, J = 11.8, 6.8 Hz, 1H), 2.59 (t, J = 7.2 Hz, 2H), 1.62–1.46 (m, 2H), 1.20–1.12 (m, 10H), 0.84 (t, J = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 165.5, 140.2, 135.7, 129.4, 120.8, 61.3, 58.0, 56.2, 35.9, 32.5, 32.2, 30.0, 29.9, 29.8, 23.2, 14.3; ESI-HRMS $(M + H)^+$ *m/z* calcd for $C_{17}H_{29}N_2O_2$ 293.2229, found 293.2299.

(*R*)-2-Amino-3-Hydroxy-*N*-(4-Octylbenzyl)Propanamide Hydrochloride 3. Compound **3** was prepared from **21** according to the same reaction procedure to that described for **1**. Yield = 88%; ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 7.00 (d, *J* = 8.0 Hz, 2H), 6.94 (d, *J* = 8.0 Hz, 2H), 4.20 (s, 2H), 3.92–3.88 (m, 1H), 3.82 (d, *J* = 12.0 Hz, 1H), 3.63 (dd, *J* = 11.8, 6.8 Hz, 1H), 2.38 (t, *J* = 7.6 Hz, 2H), 1.45–1.31 (m, 2H), 1.17–0.99 (m, 10H), 0.69 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 142.7, 134.9, 128.0, 127.9, 61.1, 55.4, 43.7, 35.9, 32.2, 31.9, 29.8, 29.5, 22.9, 14.3; ESI-HRMS $(M + H)^+$ *m/z* calcd for $C_{18}H_{31}N_2O_2$ 307.2386, found 307.2324.

(*S*)-2-Amino-3-Hydroxy-*N*-(4-Octylbenzyl)Propanamide Hydrochloride 4. Compound **4** was prepared from **22** according to the same reaction procedure to that described for **1**. Yield = 89%; ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 7.00 (d, *J* = 8.0 Hz, 2H), 6.94 (d, *J* = 8.0 Hz, 2H), 4.17 (s, 2H), 3.90–3.87 (m, 1H), 3.85 (d, *J* = 12.7 Hz, 1H), 3.65 (d, *J* = 7.5 Hz, 1H), 2.37 (t, *J* = 7.3 Hz, 2H), 1.43–1.34 (m, 2H), 1.19–1.02 (m, 10H), 0.68 (t, *J* = 6.2 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 142.7, 134.8, 129.0, 128.0, 61.2, 55.5, 43.7, 35.8, 32.2, 31.8, 29.7, 29.5, 22.9, 14.2; ESI-HRMS $(M + H)^+$ *m/z* calcd for $C_{18}H_{31}N_2O_2$ 307.2386, found 307.2372.

(*R*)-2-Amino-3-Hydroxy-*N*-(4-Octylphenethyl)Propanamide Hydrochloride 5. Compound **5** was prepared from **23** according to the same reaction procedure to that described for **1**. Yield = 92%; ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 7.11 (d, *J* = 3.0 Hz, 4H), 3.87–3.80 (m, 2H), 3.71 (dd, *J* = 12.4, 7.9 Hz, 1H), 3.56–3.37 (m, 2H), 2.86–2.73 (m, 2H), 2.56 (t, *J* = 7.6 Hz, 2H), 1.62–1.54 (m, 2H), 1.37–1.23 (m, 10H), 0.89 (t, *J* = 6.8 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 168.0, 142.2, 137.3, 129.7, 129.6, 61.8, 56.3, 42.3, 36.5, 36.0, 33.1, 30.6, 30.4, 23.7, 14.4; ESI-HRMS $(M + H)^+$ *m/z* calcd for $C_{19}H_{33}N_2O_2$ 321.2542, found 321.2533.

(*S*)-2-Amino-3-Hydroxy-*N*-(4-Octylphenethyl)Propanamide Hydrochloride 6. Compound **6** was prepared from **24** according to the same reaction procedure to that described for **1**. Yield = 95%; ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 7.11 (d, *J* = 3.5 Hz, 4H), 3.85 (dt, *J* = 7.0, 4.2 Hz, 2H), 3.71 (dd, *J* = 12.5, 8.0 Hz, 1H), 3.56–3.37 (m, 2H), 2.87–2.75 (m, 2H), 2.56 (t, *J* = 7.6 Jz, 2H), 1.63–1.54 (m, 2H), 1.35–1.27 (m, 10H), 0.89 (t, *J* = 6.8 Hz, 1H); ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 168.0, 142.2, 137.5, 129.8, 129.6, 61.8, 56.3, 42.3, 36.6, 36.0, 33.0, 32.8, 30.6, 30.4, 23.7, 14.4; ESI-HRMS $(M + H)^+$ *m/z* calcd for $C_{19}H_{33}N_2O_2$ 321.2542, found 321.2502.

(*R*)-2-Amino-3-Hydroxy-*N*-(3-(4-Octylphenyl)Propyl) Propanamide Hydrochloride 7. Compound **7** was prepared from **25** according to the same reaction procedure to that described for **1**. Yield = 87%; ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 7.08 (d, *J* = 4.3 Hz, 4H), 3.93 (dd, *J* = 7.7, 4.4 Hz, 2H), 3.87–3.76 (m, 1H), 3.25 (t, *J* = 7.0 Hz, 2H), 2.62 (dd, *J* = 14.4, 7.0 Hz, 2H), 2.55 (t, *J* = 7.6 Hz, 2H), 1.83 (dt, *J* = 14.6, 7.2 Hz, 2H), 1.63–1.53 (m, 2H), 1.34–1.26 (m, 10H), 0.89 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 168.1, 141.6, 140.0, 129.5, 129.3, 61.8, 56.3, 40.3, 36.5, 33.7, 33.1, 32.8, 32.2, 30.6, 30.4, 23.7, 14.5; ESI-HRMS $(M + H)^+$ *m/z* calcd for $C_{20}H_{35}N_2O_2$ 335.2699, found 335.2621.

(*S*)-2-Amino-3-Hydroxy-*N*-(3-(4-Octylphenyl)Propyl) Propanamide Hydrochloride 8. Compound **8** was prepared from **26** according to the same reaction procedure to that described for **1**. Yield = 84%; ^{1}H NMR (400 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 7.00 (s, 4H), 4.01–3.93 (m, 1H), 3.90 (dd, *J* = 11.9, 4.0 Hz, 1H), 3.70 (dd, *J* = 11.8, 7.0 Hz, 1H), 3.17 (t, *J* = 7.1 Hz, 2H), 2.53 (t, *J* = 7.2 Hz, 2H), 2.46 (t, *J* = 7.6 Hz, 2H), 1.82–1.70 (m, 2H), 1.48 (dd, *J* = 14.8, 7.3 Hz, 2H), 1.24–1.13 (m, 10H), 0.79 (t, *J* = 6.9 Hz, 3H); ^{13}C NMR (100 MHz, $CDCl_3$:CD_3OD = 3:1[v/v]) δ 166.6, 140.5, 138.2, 128.3, 128.0, 60.7, 55.0, 39.3, 35.4, 32.4, 31.7, 31.4, 29.4, 29.3, 29.0, 22.4, 13.4; ESI-HRMS $(M + H)^+$ *m/z* calcd for $C_{20}H_{35}N_2O_2$ 335.2699, found 335.2662.

Cell Culture and Proliferation Assays. HT29 cells were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C in a humidified 5% CO_2/95% air atmosphere. HT29 cells were seeded in 96-well plates at a density of 3×10^3 cells/100 μL/well and incubated for 24 h. The cells were then incubated in culture medium containing synthetic compounds (40 μM). Following 24 h of incubation, the cell viability was determined using a EZ-CYTOX kit according to the manufacturer's protocol.

Annexin-V Staining. Apoptosis was determined using an annexin-V FITC apoptosis detection kit, according to the manufacturer's instructions. Briefly, the cells were washed with ice-cold PBS and resuspended in binding buffer. The cell suspension was incubated with annexin-V-FITC and propidium iodide at room temperature. After incubation, stained cells were analyzed by Arthur™ Image Based Cell Analyzer.

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Supporting Information. Additional supporting information is available in the online version of this article.

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