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Synthesis and biological properties of novel sphingosine derivatives

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Abstract—Sphingosine-1-phosphate (S-1P) derivatives such as *threo*-(2*S*,3*S*)-analogues, which are C-3 stereoisomers of natural *erythro*-(2*S*,3*R*)-S-1P, have been synthesized starting from L-serine or (1*S*,2*S*)-2-amino-1-aryl-1,3-propanediols (**6**). *threo*-(1*S*,2*R*)-2-Amino-1-aryl-3-bromopropanols (HBr salt) have also been prepared from **6**. The *threo*-S-1Ps and the *threo*-amino-bromide derivatives have shown potent inhibitory activity against Ca^{2+} ion mobilization in HL60 cells induced by *erythro*-S-1P, suggesting that these compounds would compete with cell surface EDG/S1P receptors.

Sphingolipids, for example, sphingomyelin, cerebrosides, and gangliosides, are ubiquitous cell membrane components and are involved in many essential biological processes such as cell growth, cell differentiation, and adhesion.[1] Sphingolipid metabolites such as sphingosine and ceramide are emerging as a novel class of lipid second messengers.[2] Recently sphingosine-1-phosphate (S-1P), one of the metabolites, has attracted considerable attention[3] both as an intracellular second messenger[4] and as an intercellular mediator. It has been reported that S-1P binds to cell surface receptor EDG (endothelial differentiation gene)/S1P family (five subtypes: EDG-1/$S1P_1$, EDG-3/$S1P_3$, EDG-5/$S1P_2$, EDG-6/$S1P_4$, and EDG-8/$S1P_5$ have been identified[3]), which are coupled via plasma membrane G-protein to multiple effector systems.[5] Physiological significance of S-1P seems very important in the vascular system because blood platelets store S-1P abundantly and release this bioactive lipid extracellularly upon stimulation to bind surface receptors on vascular endothelial cells.[6] The receptors bound to S-1P would affect various biological responses, including mitogenesis, differentiation, proliferation, and apoptosis, and thus are supposed to be involved in a variety of pathological conditions such as angiogenesis, inflammation, and cardiovascular diseases.[7] S-1P is also suggested to be a central component of a complex network of cytokines and chemokines, which influence the responses of cells including immunosuppression.[8] Therefore, the search for agonists and antagonists toward EDG/S1P receptors would provide the basis for development of novel therapeutic agents for such diseases.[9]

Sphingoid bases are 2-amino-1,3-diols with a long-chain alkyl tail at C3. The alkyl tail may vary in chain length (from 16 to 24 carbon atoms), unsaturation (usually C4,5-*trans* olefin), and hydroxylation. The most common sphingoid base in mammalian tissues is D-*erythro*-C_{18}-sphingosine [(2*S*,3*R*,4*E*)-2-aminooctadec-4-ene-1,3-diol]. Recently, Parrill and co-workers proposed that both the C1 phosphate group and C2 ammonium moiety of D-*erythro*-S-1P are critical for its specific binding to EDG-1/$S1P_1$ receptor based on their homology modeling and point mutation studies. However, the role of the C3 hydroxy group remains to be solved.[10] Chung and co-workers reported the synthesis of four stereoisomers of S-1P and the analogues, and their binding affinities to EDG-1, -3, and -5.[11a] Their results have suggested that (1) the D-*erythro* configuration of S-1P is important for a high affinity binding, (2) the phosphate group of S-1P is essential for ligand recognition by the receptors, (3) besides the C1 phosphate group and the C2 ammonium moiety, the presence and configuration of the C3 hydroxy group of S-1P appears to be very important for specific binding.

Encouraged by these studies, we planned to investigate agonist/antagosist activities of novel S-1P analogues

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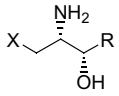
toward EDG/S1P receptors. Herein we report the synthesis of several analogues and their effects on Ca^{2+} ion mobilization in HL60 leukemia cells expressing these receptors.[12] In addition, their actions on the growth of vascular smooth muscle cells and on an inflammation model are reported.

We recently reported[13] a highly diastereoselective synthesis of both D-*erythro*- and L-*threo*-sphingosines from L-serinal derivative (Garner's aldehyde[14]) with 1-alkenyl nucleophiles prepared via hydrozirconation[15] of terminal alkynes. Thus, as shown in Scheme 1, *N*-Boc-sphingosine derivatives *erythro*-**3a**–**c** and *threo*-**3a**–**c** (**a**: natural length C18; **b**: shorter-chain homologue; **c**: styryl analogue) were prepared[13] from Garner's aldehyde (**1**) in a stereocontrolled manner via the *N*,*O*-isopropylidene acetal derivatives *erythro*-**2a**–**c** and *threo*-**2a**–**c**, respectively. Selective phosphorylation of C1 alcohol of **3** was achieved by a procedure of Bielawska and co-workers[16] with $(MeO)_3P$ and CBr_4 in pyridine.[9d,11] Treatment of the dimethyl phosphate **4** with trimethylsilyl bromide (TMSBr) followed by addition of water resulted in deprotection of both the Boc group and the phosphate dimethyl ester to afford S-1P derivative (*erythro*-**5a**–**c** and *threo*-**5a**–**c**). The spectral and physical data of these S-1P derivatives were identical with those reported[11a,16,17] and/or consistent with assigned structures.[18]

We then evaluated biological activities of the S-1P analogues by measuring Ca^{2+} ion mobilization in HL60 cells.[19] The bioassays have indicated that *erythro*-**5a**–**c** show the Ca^{2+} ion increasing activity comparable to natural S-1P, whereas *threo*-**5a**–**c** do not show that activity (data not shown). Interestingly, as shown in Table 1, *threo*-**5a**–**c** inhibit the natural S-1P induced-Ca^{2+} ion increase at rather low concentrations (IC_{50} = 0.015–0.037 μM).[20] Thus the *threo*-(2*S*,3*S*)-analogues might be recognized as a ligand by EDG/S1P receptors to show potent antagonist-like activities. Since subtype specific receptors were not used in this preliminary assay, these observations may reflect total affairs concerning with the receptors.

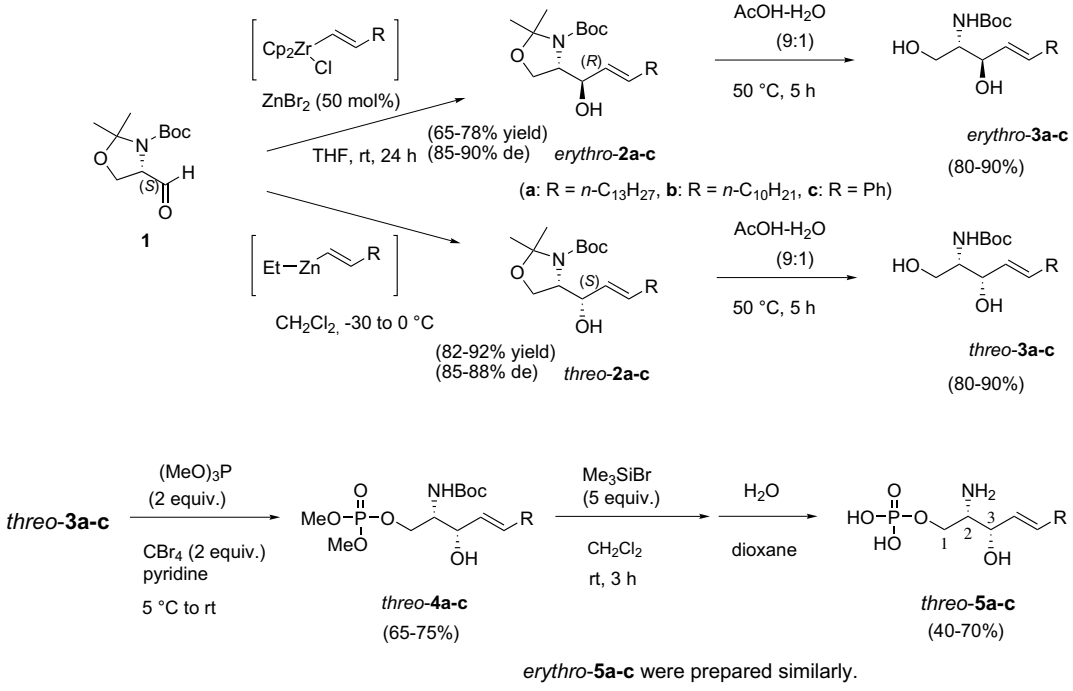
Table 1. Inhibition concentration (50%) of S-1P derivatives (*threo*-amino alcohols) against Ca^{2+} ion increase in HL60 induced by natural S-1P



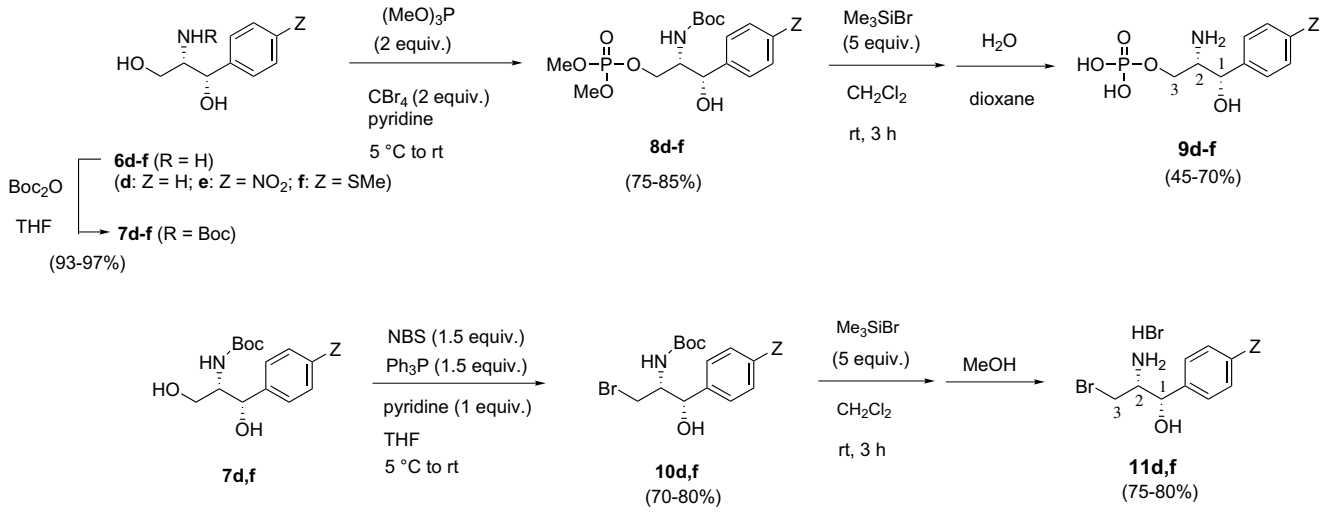
Compound	R	X	IC_{50} (μM)[a]
threo-**5a**	(*E*)-*n*-Pentadec-1-enyl	OPO_3H_2	0.031
threo-**5b**	(*E*)-*n*-Dodec-1-enyl	OPO_3H_2	0.015
threo-**5c**	(*E*)-Styryl	OPO_3H_2	0.037
9d	Phenyl	OPO_3H_2	0.179
9e	*p*-Nitrophenyl	OPO_3H_2	0.056
9f	*p*-(Methylthio)phenyl	OPO_3H_2	0.015
11d[b]	Phenyl	Br	0.071
11f[b]	*p*-(Methylthio)phenyl	Br	0.018

[a] The values are the mean of duplicate experiments.
[b] HBr salt was used.

Since the *threo*-S-1Ps showed inhibitory effect, our attention was turned to readily available *threo*-amino alcohols. Commercially available *threo*-2-amino-1-aryl-1,3-propanediols (**6d**–**f**; **d**: phenyl; **e**: *p*-nitrophenyl; **f**: *p*-methylthiophenyl) are suitable for our purpose. Phosphorylation of the primary alcohol was carried out in a



Scheme 1. Synthesis of S-1P derivatives.



Scheme 2. Synthesis of *threo*-amino alcohols.

similar manner as above. Thus, as shown in Scheme 2, treatment of **6** with di-*tert*-butyl dicarbonate gave *N*-Boc derivative **7**, which was treated with $(MeO)_3P$ and CBr_4 in pyridine to give the phosphate ester of the primary alcohol **8**. Deprotection with TMSBr afforded the amino-phosphate **9d–f**, which was purified by recrystallization from aqueous THF or by reversed phase column chromatography.[21] During the final step, a small amount of the primary bromide was formed. The bromide was soluble in water and polar organic solvents, and easily purified. Thus the bromides were prepared to evaluate their biological activities. Treatment of Boc-amino diol **7** with *N*-bromosuccinimide and triphenylphosphine gave the primary bromide **10** in good yield, which was treated with TMSBr to give the *threo*-(1*S*,2*R*)-amino-bromide HBr salt **11d,f**. These products were purified by silica gel chromatography eluting with CH_2Cl_2–MeOH.[22]

Bioassays were carried out as mentioned above. As shown in Table 1, not only the (*S*,*S*)-amino phosphate **9d–f** but also the (*S*,*R*)-amino bromide HBr salts **11d,f** inhibited the natural S-1P-mediated Ca^{2+} increase at low concentrations (IC_{50} = 0.015−0.179 μM). None of these analogues induced Ca^{2+} ion increase.

Other analogues were also examined. As depicted in Figure 1, the enantiomer of **9d** (*ent*-**9d**) and the deoxy analogues of **11d** (**12**) were prepared from commercially available (*R*,*R*)-2-amino-1-phenyl-1,3-propanediol and (*R*)-2-amino-3-phenyl-1-propanol, respectively, by the aforementioned methods. These analogues showed neither Ca^{2+} increasing activity nor its inhibitory effect.

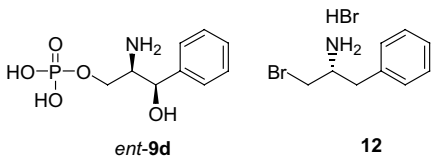


Figure 1.

Next we evaluated the actions of the S-1P analogues on the growth of vascular smooth muscle cells (SMCs). It is hypothesized that, with the progression of arteriosclerosis, vascular SMCs are transformed from the contractile type to the synthetic type, and while secreting inflammatory cytokines, the cells proliferate to cause arteriosclerotic lesions (Roth's hypothesis). SMCs are reported to proliferate in response to natural S-1P with acts on EDG/S1P receptors expressed on the cell surface.[23] Thus the actions of *threo*-S-1P analogues **9f** and **5b** on the growth of vascular SMCs were measured in the following manner. Along with natural S-1P (1.0 μM) as the growth factor, **9f** or **5b** was added to cultured SMCs,[24] and 24 h later, the cell density was measured by BrdU assay.[25] The results shown in Figure 2 have indicated that compounds **9f** and **5b** inhibit the cell growth dose dependently in concentrations from 0.01 to 0.03 μM and from 0.01 to 0.1 μM, respectively.

We also evaluated the actions on inflammation. When vascular endothelia are injured, inflammatory cytokines such as PDGF (platelet-derived growth factor) are released from the aggregated and activated platelets, and promote inflammation. Severe inflammation is

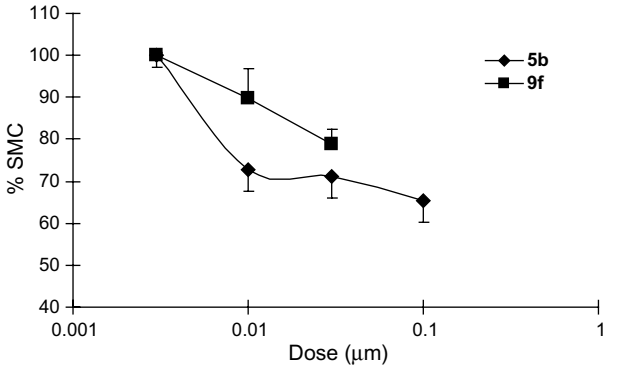


Figure 2. Effect of (*S*,*S*)-S-1P (**5b** and **9f**) on the growth of rat smooth muscle cells activated by natural S-1P. (Data are the mean ± standard error from at least two experiments.)

presumed to destroy homeostasis of cardiovascular organs and progress arteriosclerosis. Since natural S-1P is considered to have the same action as PDGF, S-1P is employed as an inflammation-inducing agent to establish a pseudo-blood vessel in vitro model. By using this model,[26] the actions of the *threo*-(*S*,*R*)-amino-bromide **11f** were examined. In the presence of **11f** (0.3–3.0 μM), the number of neutrophils transmigrating through bovine endothelial cell layer and that adhering to the cell layer decreased to 74–82% and 36–42% of the control (without **11f**), respectively. Thus the (*S*,*R*)-bromide **11f** has shown inhibitory effect on the S-1P-induced inflammation, thereby having possibility for maintaining the homeostasis of cardiovascular organs.

Receptor subtypes EDG-1/S1P$_1$ and EDG-3/S1P$_3$ are expressed in vascular endotherial cells and vascular SMCs,[7b] whereas the expression of EDG-5/S1P$_2$ is more abundant in SMCs than in endotherial cells.[9b,27] Owing to the slight difference in receptor subtypes,[28] the modes of actions of the S-1P analogues should be further elucidated.

In summary, we have synthesized sphingosine-1-phosphate derivatives such as *threo*-(*S*,*S*)-analogues starting from L-serine or (1*S*,2*S*)-2-amino-1-aryl-1,3-propanediols. Bioassays of the S-1P analogues using HL60 cells have indicated that (1) D-*threo*-(2*S*,3*S*)-S-1P homologues (*threo*-**5a**,**b**) inhibit the natural *erythro*-S-1P-mediated Ca^{2+} ion increase at low concentrations (IC_{50} = 0.015−0.031 μM), (2) the long-chain alkenyl group of *threo*-S-1P is replaced by aryl (**9d**–**f**) or styryl (*threo*-**5c**) group without significant loss of the inhibitory activity, (3) the C1 phosphate group is replaced by bromide (**11d**,**f**) without loss of the inhibitory activity, (4) Enantiomeric *threo*-(*R*,*R*)-amino alcohol (*ent*-**9d**) and deoxy-amino-bromide (**12**) do not show the activity. Therefore the presence and configuration of the amino alcohol moiety appear to be very important for the antagonist-like inhibitory effect.

Although we did not use recombinant cells overexpressing exogenous EDG/S1P receptors abundantly and further experiments are required to elucidate the relationship between the S-1P analogues and the receptors, the results described here would substantially contribute to the development of potent and selective antagonist for EDG/S1P receptors. In addition, some analogues exhibited inhibitory effects against natural S-1P-mediated responses: growth of vascular smooth muscle cells and neutrophils adhesion. Thus they might contribute therapeutic tools against vascular diseases such as arteriosclerosis.

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12. It has been reported in Ref. 5b that, when the EDG/S1P receptor on HL60 cell (prolymphoblastoma) surface is bound to S-1P, G-protein would be phosphorylated to activate IP_3 kinase, whereafter the intracellular Ca^{2+} concentration is increased.
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18. Compound *threo*-**5c**: ^{1}H NMR (CD_3OD–CD_3CO_2D = 4:1, 270 MHz) δ 3.65 (m, 1H), 4.03 (m, 1H), 4.14 (m, 2H), 6.22 (dd, J = 6.0, 16.0 Hz, 1H), 6.80 (d, J = 16.0 Hz, 1H), 7.22–7.40 (m, 5H); HRMS (CI) calcd for $C_{11}H_{17}NO_5P$ $(M+H)^+$ 274.0774, found 274.0790.
19. Assay protocol: Cell suspension, which includes Ca^{2+} chelating reagent Fura-2AM, was charged into a quartz cell, which was then mounted on a fluorometer LS50B (Perkin Elmer, for cell measurement). An excitation wavelength was alternately switched between 340 nm (for Fura-2 chelating with Ca^{2+}) and 380 nm (for unreacted Fura-2) at intervals of 0.5 ms, and fluorescence intensity at 510 nm was measured.
20. The Ca^{2+} ion increase by each test substance was measured as mentioned in Ref. 19. When fluorescence intensity was stabilized after the addition of a test substance (within a few minutes), natural S-1P (1.0 μM) was added to the cell suspension and fluorescence intensity was measured to examine the inhibitory effect of the substance against Ca^{2+} increase by natural S-1P. When the substance showed the inhibitory effect, dose dependency was examined at 0.3, 0.1, 0.03, 0.01, 0.003, and 0.001 μM, and the IC_{50} (50% inhibition concentration) value was determined from at least two independent experiments.
21. Compound **9d**: mp 244–247 °C (dec); ^{1}H NMR (CD_3OD–CD_3CO_2D = 4:1, 270 MHz) δ 3.59 (m, 1H), 3.76 (m, 1H), 4.00 (m, 1H), 4.89 (d, J = 8.5 Hz, 1H), 7.35–7.43 (m, 5H); Anal. Calcd for $C_9H_{14}NO_5P$: C, 43.73; H, 5.71; N, 5.67. Found: C, 43.64; H, 5.67; N, 5.48.
22. Compound **11f**: mp ca. 150 °C; $[\alpha]_D^{24}$ −13.0 (c 2.0, $CHCl_3$–MeOH (1:1)); ^{1}H NMR ($CDCl_3$–CD_3OD, 270 MHz) δ 2.50 (s, 3H), 3.27 (dd, J = 6.1, 12.7 Hz, 1H), 3.57 (dd, J = 3.4, 12.7 Hz, 1H), 3.57 (1H, m), 4.81 (d, J = 8.5 Hz, 1H), 7.28 (d, J = 8.5 Hz, 1H), 7.38 (d, J = 8.3 Hz, 1H); ^{13}C NMR ($CDCl_3$–CD_3OD, 67.8 MHz) δ 14.8, 29.3, 57.0, 71.6, 126.2, 126.8, 135.3, 139.5; Anal. Calcd for $C_{10}H_{15}NOSBr_2$: C, 33.63; H, 4.23; N, 3.92; S, 8.98; Br, 44.75. Found: C, 34.29; H, 4.09; N, 3.91; S, 8.91; Br, 44.62.
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26. A pseudo-blood vessel in vitro inflammation model was constructed as follows: Transwells consisting of two compartments separated by porous membrane were used. A single layer of bovine endothelial cells was cultured on the membrane at the bottom surface of the transwell upper compartment. A suspension of fluorescence-labeled neutrophils was added to the upper compartment, and natural S-1P was suspended in the lower compartment to an end concentration of 10 μM. That is, the upper compartment corresponds to the interior of the blood vessel, while the lower one corresponds to the site of inflammation outside the blood vessel. Fluorescence intensities at 530 nm were measured to determine the number of the neutrophils passing through the endothelial layer and that adhering to it.
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