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Syntheses of Sphingosine-1-phosphate Stereoisomers and Analogues and Their Interaction with EDG Receptors

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Abstract—Sphingosine-1-phosphate (S1P) is considered to be an important regulator of diverse biological processes acting as a natural ligand to EDG receptors. As a preliminary study to develop potent and selective agonist and antagonist for EDG receptors, we report synthesis of S1P stereoisomers and analogues and their binding affinities to EDG-1, -3, and -5.

Sphingosine-1-phosphate (S1P) **1a**, one of the sphingolipid metabolites, has been proposed to act as both an extracellular mediator and an intracellular second messenger. S1P-activated extracellular effects are mediated via recently identified plasma membrane G protein-coupled receptor EDG-1 (endothelial differentiation gene-1) family (EDG-1, -3, -5, -6 and -8), whereas specific intracellular targets remain to be defined.[1] These receptors are coupled differentially via G_i, G_q, $G_{12/13}$ and Rho to multiple effector systems whose signaling pathways are linked to transcription factor activation, cytoskeletal proteins, adhesion molecule expression, and caspase activities.[2] EDGs can affect diverse biological responses, including mitogenesis, differentiation, migration and apoptosis, and thus are supposed to be involved in a variety of pathological conditions such as angiogenesis, inflammation, and cardiovascular diseases, and so on (Fig. 1).[3]

Therefore, S1P analogues with different specificities and affinities for the different EDG receptors should be useful in determining which receptors mediate what specific biological responses to S1P. Identification of S1P agonists and antagonists may also provide the basis for development of novel therapeutic agents.

Recently, Parrill et al. proposed that both the C1 phosphate group and the C2 ammonium moiety of D-*erythro* S1P **1a** are critical for its specific binding to EDG-1 receptor based on their homology modeling and point mutation studies.[4] However, the role of the C3 hydroxyl group remains to be identified. Also, the syntheses and biological evaluation of stereoisomers of S1P and dihydro S1P have not yet been reported. We have now carried out the syntheses of four stereoisomers **1b–d** of S1P and their analogues, and studied their interaction with EDG receptors in attempts to evaluate their biological significance and also to develop potential agonists and antagonists for EDG receptors.

Chemistry

In the preparation of stereoisomers of S1P and dihydro S1P, our synthesis began with stereoisomers of sphingosine **2a**, that were readily obtained by the practical method recently developed in our laboratory (Scheme 1).[5] Sphingosine **2a** was converted to the *N*-Boc protected form **3a** under conventional conditions.

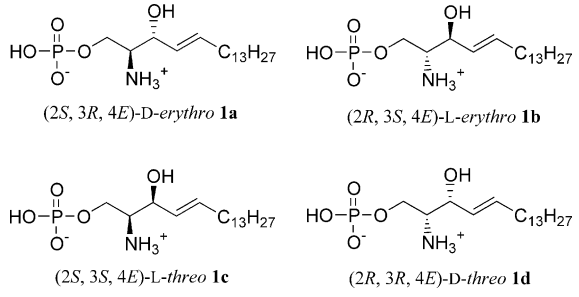

Figure 1. Structure of stereoisomers of S1P.

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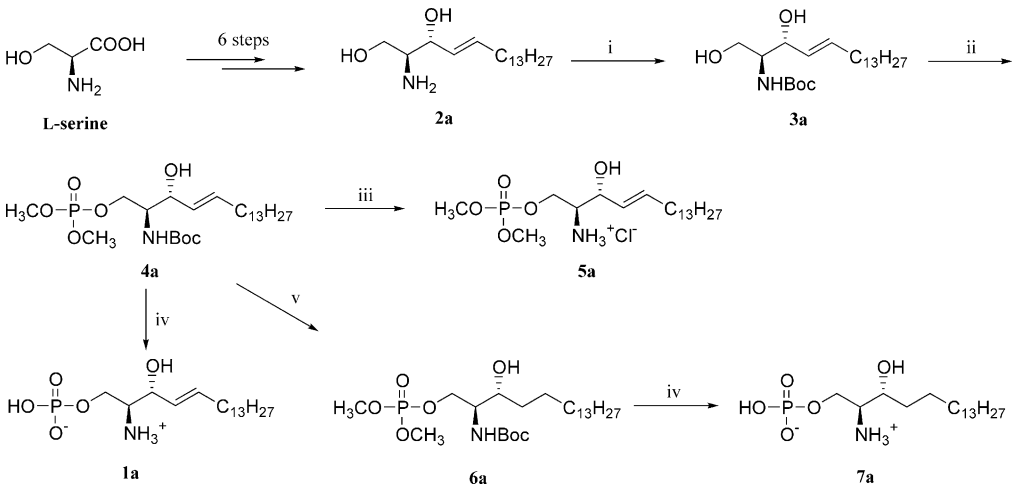


Scheme 1. Reagents and conditions: (i) Boc_2O/Et_3N, MeOH, 40 °C, overnight, 95%; (ii) $P(OCH_3)_3/CBr_4$, pyridine, 0 °C, 2 h, 83%; (iii) TMSCl, MeOH, rt, 6 h, 64%; (iv) TMSBr, CH_2Cl_2, 0 °C, 2 h, 63%; (v) H_2, 10% Pd/C, EtOAc, rt, 12 h, 92%.

Phosphorylation of **3a** with trimethyl phosphite afforded the C1 phosphorylated sphingosine **4a** following the method described by Szulc et al.[6a] Under these conditions, monophosphorylated product **4a** was prepared in 83% yield, together with 9% of diphosphorylated compound which was easily separated by column chromatography. TMSBr-mediated deprotections of the phosphate ester and the *N*-Boc group of **4a** gave S1P **1a** in 63% yield.[7] When TMSCl was used instead, dimethyl ester **5a** was produced exclusively.[8] Hydrogenation of **4a**, followed by deprotections gave rise to dihydro S1P **7a**.[7] By employing the same procedures on the other stereoisomers of sphingosine, the corresponding stereoisomers of S1P and dihydro S1P were prepared (Table 1).

The C3 keto analogues of S1P were synthesized from C3 keto sphingosine **8a**, an intermediate obtained in the course of the sphingosine synthesis.[5] C3 Keto analogues **10a**,**b** of S1P were generally obtained in low yields due to their instability and difficulty involved in purification (Scheme 2).

We synthesized C3 deoxy version **16a** of dihydro S1P instead of C3 deoxy S1P for the synthetic easiness and structural similarity. The synthesis commenced with the known Garner aldehyde which was readily available from serine.[9] The Wittig reaction of Garnar aldehyde **11a** with pentadecylphosphonium bromide in the presence of LHMDS provided olefin **12a** as a mixture of *E* and *Z* isomers. Hydrogenation of olefin **12a**, followed by deprotection in 90% aq acetic acid led to the alcohol **14a**. Phosphorylation of **14a** and subsequent deprotections afforded the desired C3 deoxy analogue **16a** of S1P (Scheme 3). Preparation of its enantiomer **16b** was similarly carried out from D-serine.

Synthesis of the C3 amide analogues of S1P was started from serine. L-Serine was protected with *N*-Boc and then treated with tetradecylamine in the presence of DIC and HOBT to generate amide **18a**. Amide **18a** was phosphorylated and then deprotected to give the desired C3 amide analogue **20a** of S1P (Scheme 4).

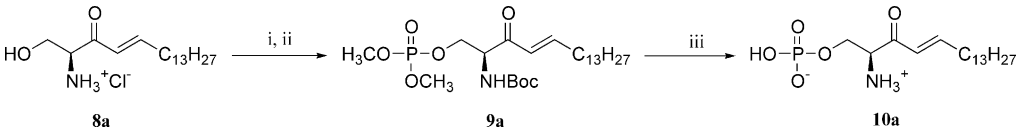
Table 1. Physical properties of S1P stereoisomers and their analogues

Compd	$[\alpha]_D^{20}$ (AcOH)	Mp (°C)	^{31}P (δ, ppm)[a]
1a	−1.22 (c 0.40)	>143 (dec.)	3.56
1b	+4.84 (c 0.37)	>149 (dec.)	3.53
1c	−27.38 (c 0.22)	>144 (dec.)	3.51
1d	+29.04 (c 0.21)	>151 (dec.)	4.11
5a	−2.61 (c 0.34)[b]	79-81	3.58
7a	−3.04 (c 0.25)	>180 (dec.)	4.00
7b	+6.90 (c 0.29)	>181 (dec.)	3.99
7c	−17.58 (c 0.24)	>179 (dec.)	4.10
7d	+18.34 (c 0.26)	>177 (dec.)	4.08
10a	ND[c]	>87 (dec.)	ND[c]
10b	ND[c]	>89 (dec.)	5.96
16a	−7.80 (c 0.26)	>155 (dec.)	3.51
16b	+9.76 (c 0.33)	>153 (dec.)	4.17
20a	−2.27 (c 0.34)	>180 (dec.)	2.82
20b	+5.34 (c 0.36)	>182 (dec.)	2.73

[a]External reference, 80% H_3PO_4 in D_2O.
[b]Measured in $CHCl_3$.
[c]Not determined.

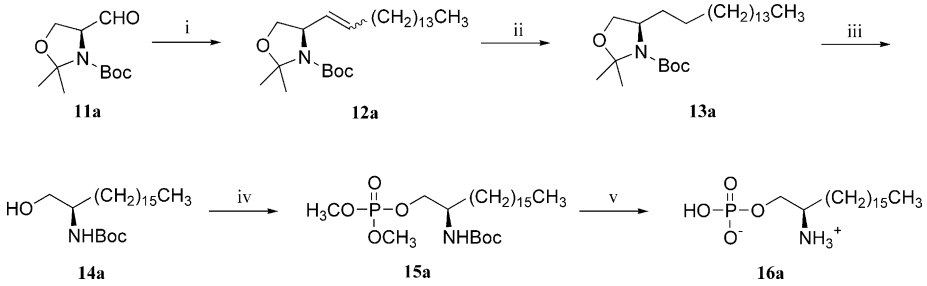
Results and Discussion

The synthetic stereoisomers of S1P and their analogues were evaluated in vitro for the EDG receptors binding affinity by measuring their ability to displace radioligand, [^{3}H]-S1P from EDG receptors (EDG-1,-3, and -5) which were expressed in CHO cells (Fig. 2).[10,11] As was reported previously,[12] D-*erythro* dihydro S1P (sphinganine-1-phosphate) **7a**, which lacks the double bond at the 4 position, blocked the S1P binding to EDG-1, -3, and -5 (41, 86, and 54%, respectively). L-*Erythro* S1P **1b**, the enantiomer of L-*erythro* S1P, showed affinity low to EDG-1 (7.4%), moderate to EDG-3 (20%), and relatively good to EDG-5 (41%), whereas L-*erythro* dihydro S1P **7b** did not compete with [^{3}H]-S1P for binding to EDGs at all. Both L-*threo* S1P **1c** and L-*threo* dihydro S1P **7c**, which have opposite configuration only at the C3 OH of D-*erythro* S1P **1a**,



8a **9a** **10a**

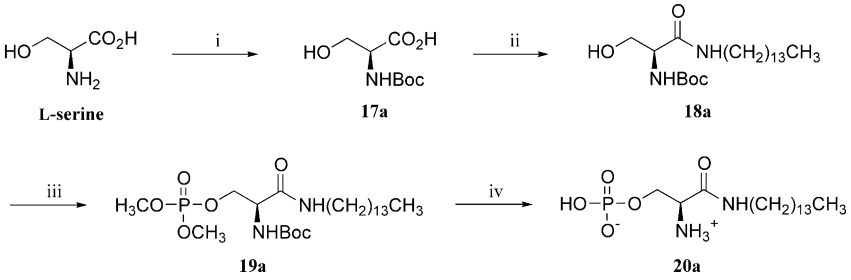
Scheme 2. Reagents and conditions: (i) Boc_2O/Et_3N, MeOH, 40 °C, overnight, 93%; (ii) $P(OCH_3)_3$, CBr_4, pyridine, 0 °C, 2 h, 87%; (iii) TMSBr, CH_2Cl_2, 0 °C, 2 h, 29%.



11a **12a** **13a**

14a **15a** **16a**

Scheme 3. Reagents and conditions: (i) $Ph_3P^+C_{15}H_{31}Br^-$/LHMDS, THF, −78 °C∼rt, overnight, 91%; (ii) H_2, 10% Pd/C, EtOAc, rt, 5 h, 96%; (iii) LiCl, 90% aq AcOH, rt, overnight, 87%; (iv) $P(OCH_3)_3$, CBr_4, pyridine, 0 °C, 2 h, 84%; (v) TMSBr, CH_2Cl_2, 0 °C, 2 h, 58%.



L-serine **17a** **18a**

19a **20a**

Scheme 4. Reagents and conditions: (i) Boc_2O, 1 N NaOH, dioxane–H_2O, rt, 6 h; (ii) $H_2N(CH_2)_{13}CH_3$, DIC, HOBT, CH_2Cl_2, rt, overnight; (iii) $P(OCH_3)_3$, CBr_4, pyridine, 0 °C, 4 h, 53% for three steps; (iv) TMSBr, CH_3CN, rt, 4 h, 62%.

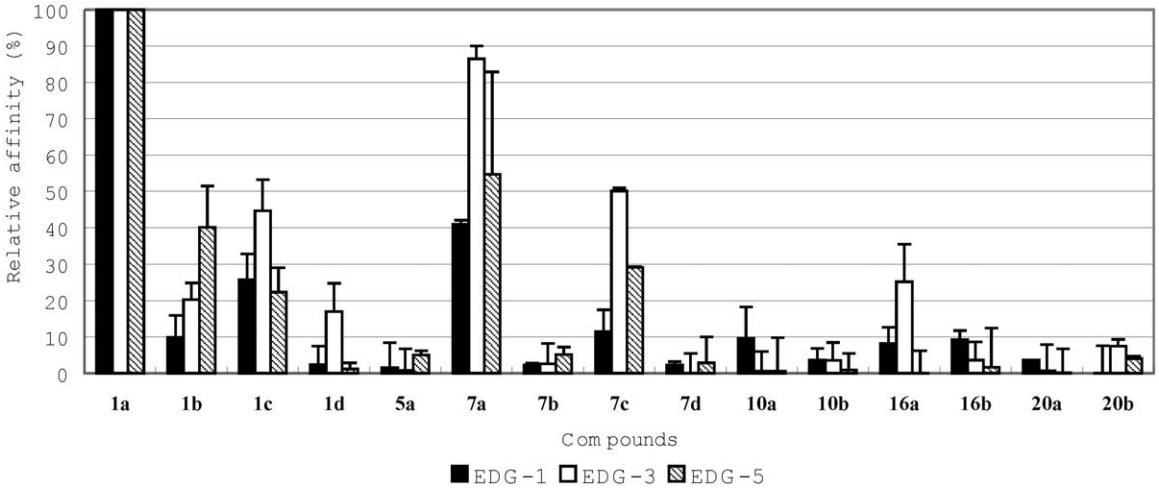


Figure 2. Relative binding affinity: competition by S1P isomers and analogues for specific binding of [^{3}H]-S1P to EDG-1, -3, and -5 receptors. CHO cells transfected with *edg*-1, -3, and -5, respectively were incubated in the presence of 2 nM [^{3}H]-S1P without or with 100 nM of the indicated compounds. Results are means ± standard deviation of duplicate determinations.

showed relatively high affinity to EDGs (for L-*threo* S1P **1c**, 27, 46, and 23% to EDG-1, -3, and -5; for L-*threo* dihydro S1P **7c**, 12, 51, and 30% to EDG-1, -3, and -5, respectively), whereas D-*threo* forms of S1P and dihydro S1P had no significant effect on specific [^{3}H]-S1P binding to EDGs. D-*Erythro* S1P dimethyl ester **5a** was completely inactive as expected.

On the other hand, the S1P analogues which were modified at the C3 position of S1P (C3 keto, C3 deoxy, and C3 amide analogues) did not effectively bind to EDGs except that (2*R*) C3 deoxy dihydro S1P **16a** showed 29% of affinity to EDG-3 comparing with dihydro S1P **7a**, and (2*S*) C3 keto S1P **10a** and (2*S*) C3 deoxy dihydro S1P **16b** showed weak affinity to

only EDG-1. It indicates that the C3 hydroxyl group of S1P as well as the C1 phosphate group and the C2 ammonium moiety are crucial for its binding to EDG receptors.

In summary, we have synthesized all four stereoisomers of S1P and dihydro S1P, and their analogues which are modified at the C3 position and evaluated their binding affinity to EDG receptors for the first time. On the basis of our experimental and computational data,[13] it might be concluded that (1) the D-*erythro* configuration of S1P is important for a high affinity binding to EDGs, (2) the phosphate group of S1P is essential for ligand recognition of EDGs, (3) besides the C1 phosphate group and the C2 ammonium moiety of S1P, the presence and configuration of the C3 hydroxyl group of S1P appears to be very important for specific binding to EDGs. These insights and findings will substantially contribute to developing potent and selective agonist and antagonist for EDG receptors. Further works along these lines are currently in progress.

Acknowledgements

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7. Compounds **1a** and **7a** were identical with the reported data with respect to TLC, NMR (^{1}H, ^{13}C and ^{31}P) and FAB-MS.[6b,c]
8. Compound **5a**: $[\alpha]_D^{20}$ $-2.61°$ (*c* 0.34, $CHCl_3$), mp 79–81 °C (lit.[6a] $[\alpha]_D^{24}$ $-4.0°$ (*c* 1.0, $CHCl_3$), mp 82–83 °C).
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11. Assay protocol: [^{3}H]-S1P was purchased from American Radiolabeled Chemicals, Inc. CHO cells (CHO/Edg-1, CHO/Edg-3, CHO/Edg-5, and CHO/Mock)[14] in confluent six multiplates were washed twice with the ice-cold binding buffer consisting of 20 mM Tris–HCl (pH 7.5), 100 mM NaCl, 15 mM NaF, and 0.4% (w/v) BSA, and then incubated with the same buffer containing 2 nM [^{3}H]-S1P (about 40,000 dpm per well) and 100 nM of S1P or S1P analogues in a final volume of 0.5 mL. The plates were kept on ice for 90 min, and the cells were washed twice with the same ice-cold binding buffer to remove unbounded ligand. The cells were solubilized with the solubilizing solution composed of 0.1% SDS, 0.4% NaOH, and 2% Na_2CO_3, and the radioactivity was measured by a liquid scintillation counter after the addition of scintillation cocktail solution. The relative binding affinity of each analogues to each EDG receptors (EDG-1,-3 and-5) were presented as percentage to S1P.
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13. The homology model of EDG-1 was developed using Modeller4 (Laboratory of Molecular Biophysics in Rockefeller University, New York, USA) and Insight II program (Accelrys, San Diego, CA, USA) on a Silicon Graphics Octane workstation (1.2GB RAM, IRIX 6.5). The EDG-1 sequence was obtained from GenBank™ (AAF43420) and the bovine rhodopsin model composed of 7 transmembrane helices (PDB code 1boj) was used as a template structure for homology modeling. The results of docking studies with S1P isomers and analogues will be reported in due course.
14. CHO cells used in our bioassays were provided by Dr. Yoshiko Banno (Gifu University, Japan) and Dr. Yoh Takuwa (Kanazawa University, Japan), and we wish to thank them.