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Synthesis of Fluorescence-Labeled Sphingosine and Sphingosine 1-Phosphate; Effective Tools for Sphingosine and Sphingosine 1-Phosphate Behavior

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Abstract—A fluorescence-labeled sphingosine and sphingosine 1-phosphate have been successfully synthesized from the oxazolidinone methyl ester derived from glycidol via monoalkylation and the stereoselective reduction of the resulting ketone. The labeled sphingosine was converted into its phosphate by treatment with sphingosine kinase 1 (SPHK1) from mouse, and in platelets, and it was incorporated into the Chinese Hamster Ovarian (CHO) cells. In addition, MAPK was activated by NBD-Sph-1-P through Edg-1, Sph-1-P receptor.

Sphingolipids are known as a lipid second messenger in mammalian cells and cell membranes, and a great deal of attention has been devoted to the studies of the biological process regulated by sphingolipids (Fig. 1).[1]

Now, it has been well accepted that the sphingolipids play key roles in the cellular signal transmission pathway. Among these sphingolipids, sphingosine 1-phosphate is regarded as a new remarkable phospholipid based on the discovery of its biological receptor[2] in addition to its quite attractive biological activities. Thus, sphingosine 1-phosphate stimulates DNA synthesis, cell division, cell growth,[3] and platelet activation.[4] Meanwhile, the fluorescence-labeled sphingolipids have contributed in many areas as a tool to clarify the mode of biological action of sphingolipids. Thus, nitrobenzo-2-oxa-1,3-diazole(NBD)-labeled sphingomyelin and ceramide in which the fluorescent group is linked to the *N*-acyl terminus, are now commercially available[5] and have been widely used.[6] These compounds, however, cannot be utilized in the research of the important sphingosine and sphingosine-1-phosphate metabolism, because sphingosine is biologically produced from ceramide by hydrolysis of the *N*-acyl group, and sphingosine 1-phosphate is generated from sphingosine by phosphorylation. We now report the synthesis of newly designed fluorescence labeled analogues **1** and **2**, in which the fluorescent NBD group was situated in the terminal of the sphingosine backbone. We also describe their adaptability to sphingosine kinase and sphingosine 1-phosphate receptor as a substrate or a ligand (Fig. 2).

We previously prepared both enantiomers of 4-methoxycarbonyloxazolidinone **7** as a chiral building block from the enantiomerically pure glycidol, and succeeded in the stereoselective syntheses of γ-hydroxy-β-aminoalcohols, sphingolipids including the short-chain sphingomyelin, and azasugars by monoalkylation of the ester **7** followed by highly *syn*- and *anti*-selective reduction of the resulting ketones with L-selectride or diisobutylaluminum 2,6-di-*t*-butyl-4-methylphenoxide, respectively.[7] These methods were then used for the synthesis of the fluorescence labeled sphingolipids **1** and **2**.

The alkyl chain **6** as a backbone component was prepared starting from 1,10-decanediol **3** as shown in Scheme 1. Protection of the hydroxyl group as the THP ether followed by the Swern oxidation gave aldehyde **4**, which was converted into the desired acetylene **6** by utilizing Corey's method in two steps;[8] treatment with

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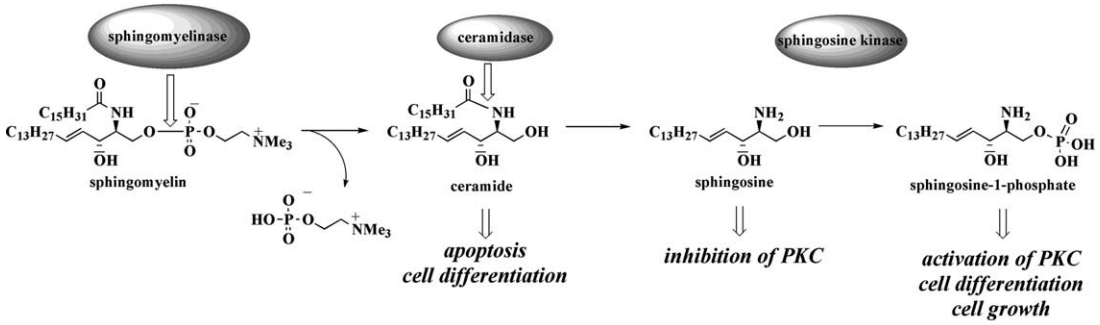


Figure 1. Sphingomyelin metabolism.

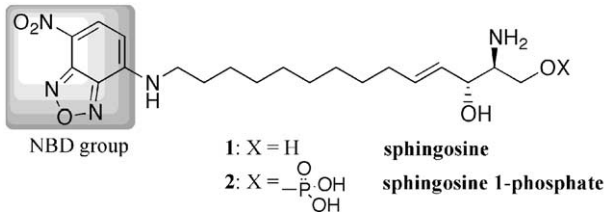
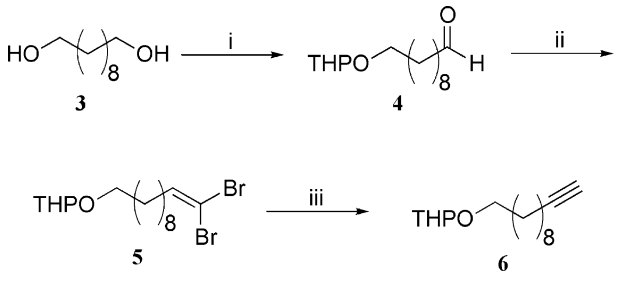


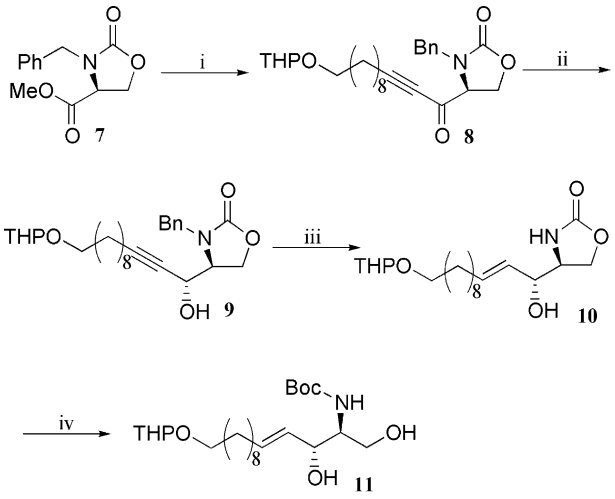
Figure 2. Designed NBD-labeled sphingolipids.

triphenylphosphine and carbon tetrabromide in the presence of triethylamine[9] followed by a reaction with 3 equivalents of *n*-butyllithium at −78 °C produced the desired acetylene **6** (Scheme 1).

The reaction of the lithium anion derived from **6** with (*S*)-(−)-ester **7** at the temperature below −100 °C in THF selectively gave ketone **8** without any dialkylated products (Scheme 2). The stereoselective reduction of the obtained ketone was successfully achieved using diisobutylaluminum 2,6-di-*tert*-butyl-4-methylphenoxide[10] to produce the desired *anti* aminoalcohol **9** in 99% yield, and the selectivity between the *anti* and *syn* was confirmed by ^{1}H NMR analysis to be 17:1. The desired acetylene **9** was then treated with Li in liquid NH_3 to produce oxazolidinone **10** in 96% yield resulting from the E olefin preparation accompanied by removal of the benzyl group. Hydrolysis of the oxazolidinone ring of **10** with potassium hydroxide in ethanol under reflux smoothly proceeded, and the subsequent protection of the produced amine selectively afforded the desired **11** by treatment with Boc_2O in H_2O and THF (50%) without isolation of the intermediary primary amine, whose isolation was difficult because of its strong polarity.



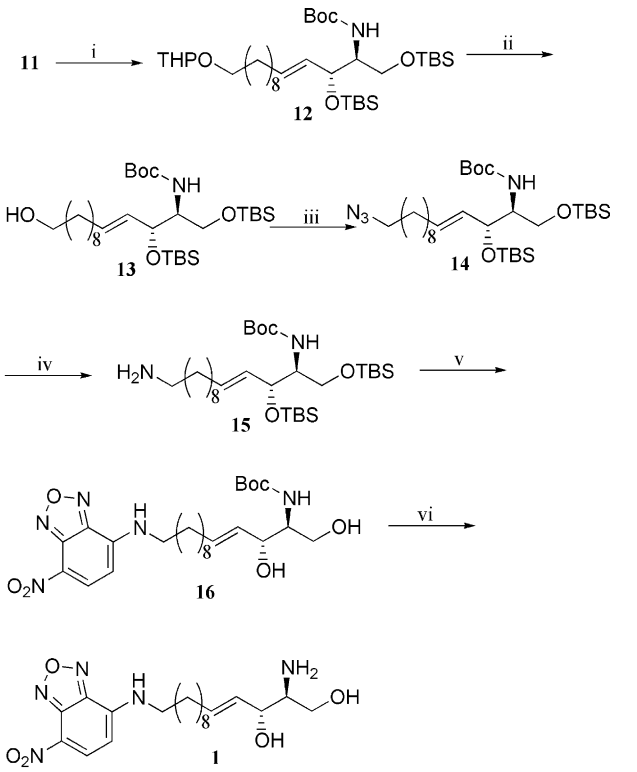
Scheme 1. Synthesis of acetylene. Reagents and conditions; (i) (a) DHP, PPTS, $CHCl_3$, 66%; (b) $(COCl)_2$, DMSO, Et_3N, CH_2Cl_2, −78 °C, 99%; (ii) PPh_3, CBr_4, CH_2Cl_2, 84%; (iii) *n*-BuLi, THF, −78 °C, quant.



Scheme 2. Synthesis of sphingosine backbone. Reagents and conditions; (i) **6**, *n*-BuLi, THF, −100 °C, 93%; (ii) diisobutylaluminum 2,6-di-*t*-butyl-4-methylphenoxide, toluene, 0 °C, 99%; (iii) Li, NH_3, THF, reflux, 96%; (iv) (a) KOH, EtOH, reflux; (b) Boc_2O, K_2CO_3, THF, H_2O, 0 °C, 84% for two steps.

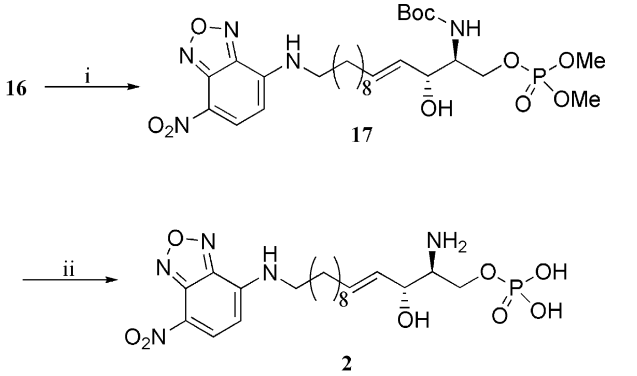
Introduction of the NBD group at the terminal position was successfully realized as follows. The two hydroxy groups of **11** were protected as *tert*-butyldimethylsilyl ethers and then removal of the THP protecting group was completed by treatment with excess $MgBr_2$[11] to produce **13**, whose terminal alcohol was activated as its mesylate. The obtained mesylate was reacted with NaN_3 in DMF to introduce an azide group. Reduction of the azide with PPh_3 in 10% aqueous THF at 60 °C proceeded smoothly to afford the corresponding terminal amine **15**. A fluorescent NBD group was successfully introduced by treatment with NBDCl in the presence of Et_3N, and the obtained compound was purified after removal of the two silyl groups to furnish the Boc protected fluorescent sphingosine **16**. Fortunately, this fluorescent compound was stable enough in a bright laboratory. Finally, deprotection of the amine with trifluoroacetic acid gave the title compound **1**,[12] which was purified by reverse phase HPLC. The ^{1}H NMR spectrum of the triacetate derived from the compound **1** was quite similar to those of natural sphingosine except for the signals of the terminal group (Scheme 3).[13]

Next, the synthesis of the labeled sphingosine 1-phosphate was accomplished according to the literature procedure.[14] Thus, **16** was treated with carbon tetrabromide



Scheme 3. Synthesis of the NBD-labeled sphingosine. Reagents and conditions; (i) TBSCl, imid., DMF; (ii) $MgBr_2$, Et_2O, 82% for two steps; (iii) (a) MsCl, Et_3N, THF; (b) NaN_3, DMF, 50 °C, 89% for two steps; (iv) PPh_3, 10% aq THF, 60 °C; (v) (a) NBDCl, Et_3N, THF; (b) 2N-HCl, MeOH, 82% for three steps; (vi) TFA, CH_2Cl_2, 33%.

and trimethylphosphite in pyridine at −10 °C to furnish the dimethyl phosphonic ester **17**. When this reaction was carried out at 0 °C, the secondly hydroxyl group of **16** was also phosphorylated. Finally, deprotection of the amino group and hydrolysis of the methyl ester were achieved by treatment with bromotrimethylsilane in acetonitrile to give sphingosine 1-phosphate **2**,[15] which was purified by reversed phase HPLC. The ^1H NMR spectrum of the obtained compound was quite similar to those of the natural sphingosine 1-phosphate except for the signals of the terminal group (Scheme 4).[16] Thus, the fluorescent NBD labeled sphingosine **1** and sphingosine 1-phosphate **2** were stereoselectively synthesized.



Scheme 4. Synthesis of the NBD-labeled sphingosine 1-phosphate. Reagents and conditions; (i) CBr_4, $P(OMe)_3$, pyr. −10 °C, 82%; (ii) TMSBr, CH_3CN, 71%.

The synthesized NBD-sphingosine was phosphorylated by recombinant mouse sphingosine kinase 1 or sphingosine kinase from human platelet lysates, yielding NBD-sphingosine 1-phosphate under the conditions applied to sphingosine phosphorylation.[17] Thus, NBD-sphingosine, like sphingosine, was recognized as a substrate for sphingosine kinase. Next, we examined the changes of intracellular distribution of NBD-sphingosine and its metabolites in the Chinese Hamster Ovarian (CHO) cells. In these experiments, cells were incubated with 1 μM NBD-sphingosine at 37 °C for varying times. NBD-sphingosine was rapidly incorporated into cells, and most of intracellular NBD-fluorescence was incorporated into cytoplasm in 5-min incubation, and to the Golgi apparatus in 15-min incubation (Fig. 3A). We examined the metabolic changes of the incorporated NBD-sphingosine in CHO cells using thin-layer chromatography (Butanol/Acetic acid/H_2O = 3:1:1). NBD-sphingosine was converted to NBD-sphingosine 1-phosphate, NBD-ceramide and NBD-sphingomyelin (Fig. 3B). The pattern of metabolic changes of NBD-sphingosine was similar to that of ^3H-sphingosine (data not shown). Based on these experiments, we concluded that NBD-sphingosine could be a useful tool to visualize the sphingosine dynamics in the cells under the fluorescent microscope.

In addition, we examined whether MAPK was activated by NBD-Sph-1-P according to the method described previously.[18] Briefly, Edg-1 expressing CHO cells (8×10^5) were cultured in serum free Ham F-12 medium for 24 h followed by incubation in the absence or presence of 500 ng/mL pertusis toxin (PTx) for 6 h. Then, the cells were stimulated with 100 nM Sph-1-P of 1 nM NBD-Sph-1-P for 5 min. Total cell lysates were prepared using lysis buffer (50 mM Tris–HCl (pH 7.5), 500 mM NaCl, 0.1% SDS, 0.5% sodium deoxycholate, 1% Triton X-100, 10 mM $MgCl_2$, protease inhibitors cocktail[19] (complete EDTA free) and 5 mM EDTA). These lysates were resuspended in Laemmli sample buffer[20] followed by boiling for 3 min. Then these samples were subjected to SDS-PAGE, and analyzed by Western

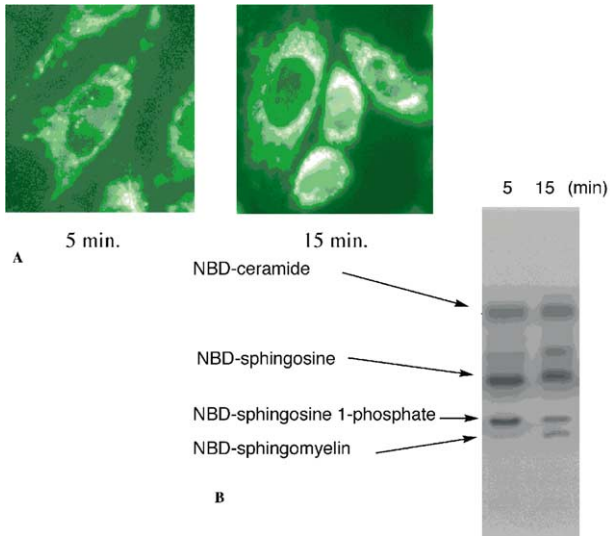


Figure 3. Incorporation into the CHO cells.

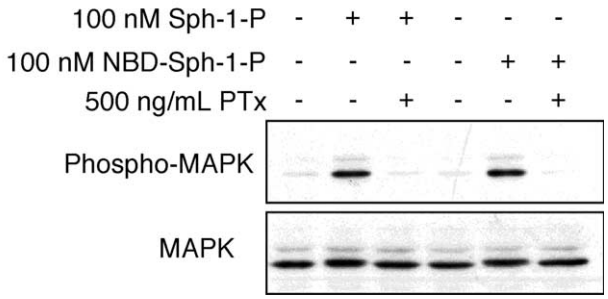


Figure 4. MAPK activation by Sph-1-P and NBD-Sph-1-P.

blotting using an anti-MAPK antibody[19] and an anti-phospho MAPK antibody.[19] These results clearly showed that NBD-Sph-1-P as well as Sph-1-P induced MAPK activation through the Edg-1 in a PTx sensitive manner. Based upon these results, we concluded that NBD-Sph-1-P is recognized as a ligand by Sph-1-P receptor, Edg-1 (Fig. 4)

In conclusion, the NBD labeled sphingosine **1** and sphingosine 1-phosphate **2** were synthesized from (−)-4-methoxycarbonyloxazolidinone. The synthesized **1** was recognized as a substrate by sphingosine kinase, and was converted into the corresponding 1-phosphate **2**. In addition, **2** was recognized as a ligand by Edg-1, Sph-1-P receptor. Thus, **1** and **2** would be useful tools to visualize the sphingosine and sphingosine 1-phosphate dynamics, respectively.

Acknowledgements

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