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Synthesis and evaluation of novel phosphate ester analogs as neutral sphingomyelinase inhibitors

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ABSTRACT

A novel sphingomyelin inhibitor RY221B-a, which contains a bipyridyl moiety as a metal coordination site was designed based upon the mechanism of phosphate ester hydrolysis. RY221B-a was synthesized from *N*-Boc-sphingosine in three steps via selective etherification using stannyl acetal. Synthesized RY221B-a exhibited relatively-strong inhibitory activity against *Bc*-SMase (IC_{50} = 1.2 μM).

Hydrolase-mediated hydrolysis of phosphate esters are fundamental reactions in a variety of important biological processes such as signal transduction, molecular recognition, energy transduction, regulation of protein function and metabolism. Sphingomyelinase (SMase[1], EC 3.1.4.12) hydrolyzes the phosphate ester-bond of sphingomyelin to generate phosphocholine and ceramide. This hydrolysis reaction is important for lipid metabolism, regulation of the immune mechanism and differentiation of cells, including angiogenesis (Fig. 1).[2]

Therefore, an inhibitor of SMase might be expected to modulate a wide variety of biological responses. So far, a number of naturally occurring products showing SMase inhibition have been reported.[3] Several synthetic inhibitors mimicking the phosphate ester moiety by substitution with stable functional groups such as a carbamate analog, carbon-analog, and a fluorinated carbon-analog have also been developed.[4] A detailed mechanism for the hydrolysis reaction mediated by SMase based on mutagenesis studies have been elucidated.[5,6] Obama et al. reported that Glu-53 of *Bc*-SMase has a crucial function for a ligand for Mg^{2+} and SMase activity.[5d] In 2006, the Sakurai group elucidated the three-dimensional structure of *Bc*-SMase from *Bacillus cereus*.[7] Analysis of the structure suggested that the ionic bond between Glu-53 and the divalent ion such as Mg^{2+} plays an important role in hydrolysis of sphingomyelin, and the Mg^{2+} ion is coordinated with oxygen atoms in the phosphate ester of sphingomyelin, which controls the location of the substrate (Fig. 2).[7] We focused on the metal ion mediated-coordination between the enzyme

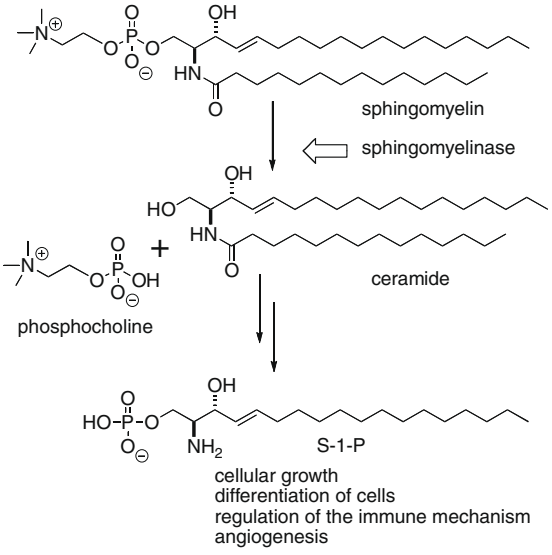

Figure 1. The role of SMase during lipid metabolism.

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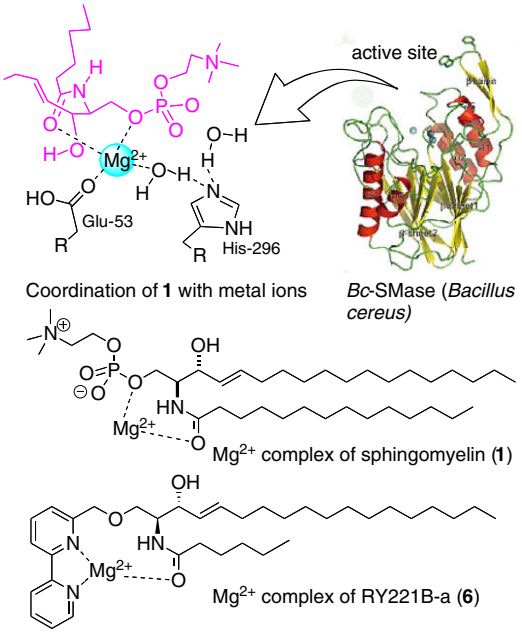
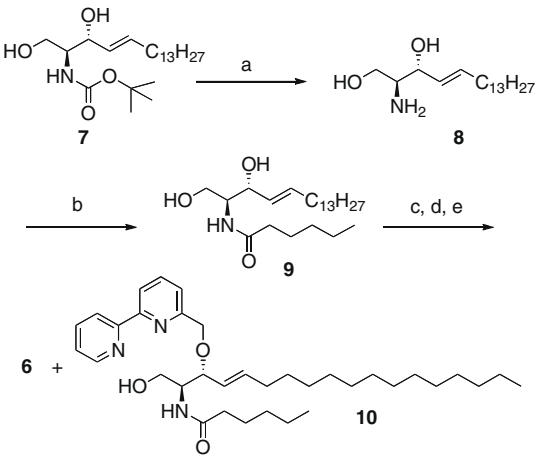


Figure 2. Design of a novel SMase inhibitor based upon substrate coordination in the active site of the enzyme.

and sphingomyelin. If the phosphate ester moiety acts as a ligand for the Mg^{2+} ion, the functional group having high coordinating ability with divalent metal ions will behave as an analog for the phosphate ester of sphingomyelin during the hydrolysis reaction. From these structural and mechanistic considerations, we designed a novel sphingomyelin analog RY221B-a (**6**) having a bipyridine moiety that acts as an efficient coordination site (Fig. 2).

The synthesis of **6** commenced with the preparation of *N*-Boc-sphingosine (**7**) from serine methyl ester in five steps according to the Suzuki protocol.[8] Deprotection of **7** was achieved by using 1 M HCl. The resulting sphingosine (**8**) was treated with hexanoyl chloride in the presence of Et_3N in CH_2Cl_2 to provide ceramide **9** in 68% yield. The introduction of bipyridyl methyl ether into ceramide **9** was achieved by the etherification method via stannyl acetal[9,10] to give RY221B-a (**6**) along with isomer (RY221B-b, **10**) after HPLC separation in 53% and 18%, yield respectively (Scheme 1).



Scheme 1. Preparation of the novel sphingomyelin analog **6**. Reagents and conditions: (a) HCl, THF, 50 °C, 4 h, 96%; (b) hexanoyl chloride (1.0 equiv), DMAP, Et_3N, CH_2Cl_2, 0 °C, 15 min, 68%; (c) Bu_2SnO (1.0 equiv), MeOH, reflux, 6 h; (d) 6-(bromomethyl)-2,2′-bipyridine (1.2 equiv), *n*-Bu_4NF (1.2 equiv), DMF, rt, 18 h; (e) HPLC separation. Compound **6**/**10** (3:1, total 71% yield).

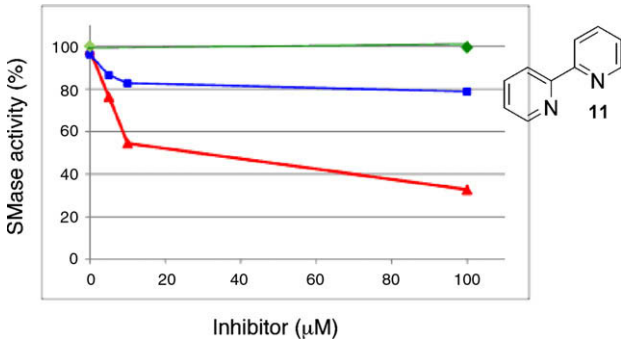


Figure 3. Inhibitory effect of **6** (trigona), **10** (square) and **11** (argyle) for the degradation of sphingomyeline liposome induced by *Bc*-SMase.

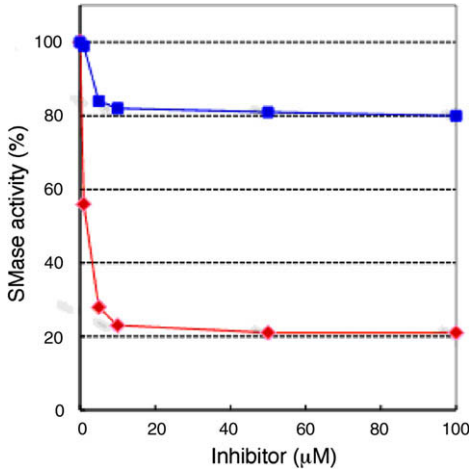


Figure 4. Inhibitory effect of **6** (argyle) and **10** (square) for the hydrolysis of ^{14}C-sphingomyeline mediated by *Bc*-SMase.

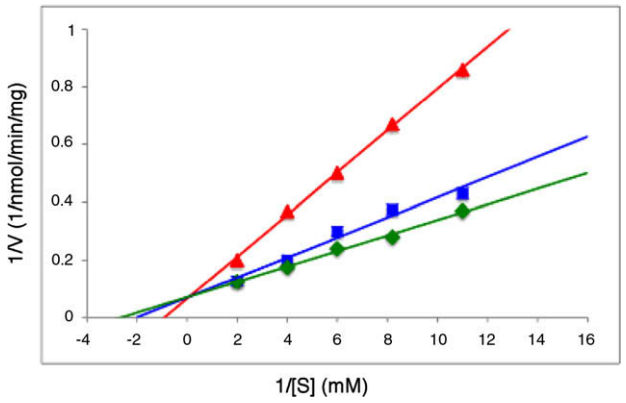


Figure 5. Lineweaver–Burk plot analysis for **6**. trigona: only *Bc*-SMase with 10 μM of **6**. Square: *Bc*-SMase with 5 μM of **6**, argyle: *Bc*-SMase. In all cases, 40 ng/mL of *Bc*-SMase was used.

The sphingomyelin liposome-disruption assay was used to evaluate the synthetic compound **6** and its isomer **10** as potential SMase inhibitors.[7,11] Compound **6** was shown to display a significant inhibitory effect against *Bc*-SMase in a concentration-dependent manner (Fig. 3). By contrast, **10** showed only weak inhibitory activity and bipyridine (**11**) displayed no detectable inhibitory action.

The detailed inhibitory activity of **6** and **10** against *Bc*-SMase were elucidated using [^{14}C]-labeled SM.[7] The IC_{50} of **6** was determined to be 1.2 μM, which is classified as one of the most potent

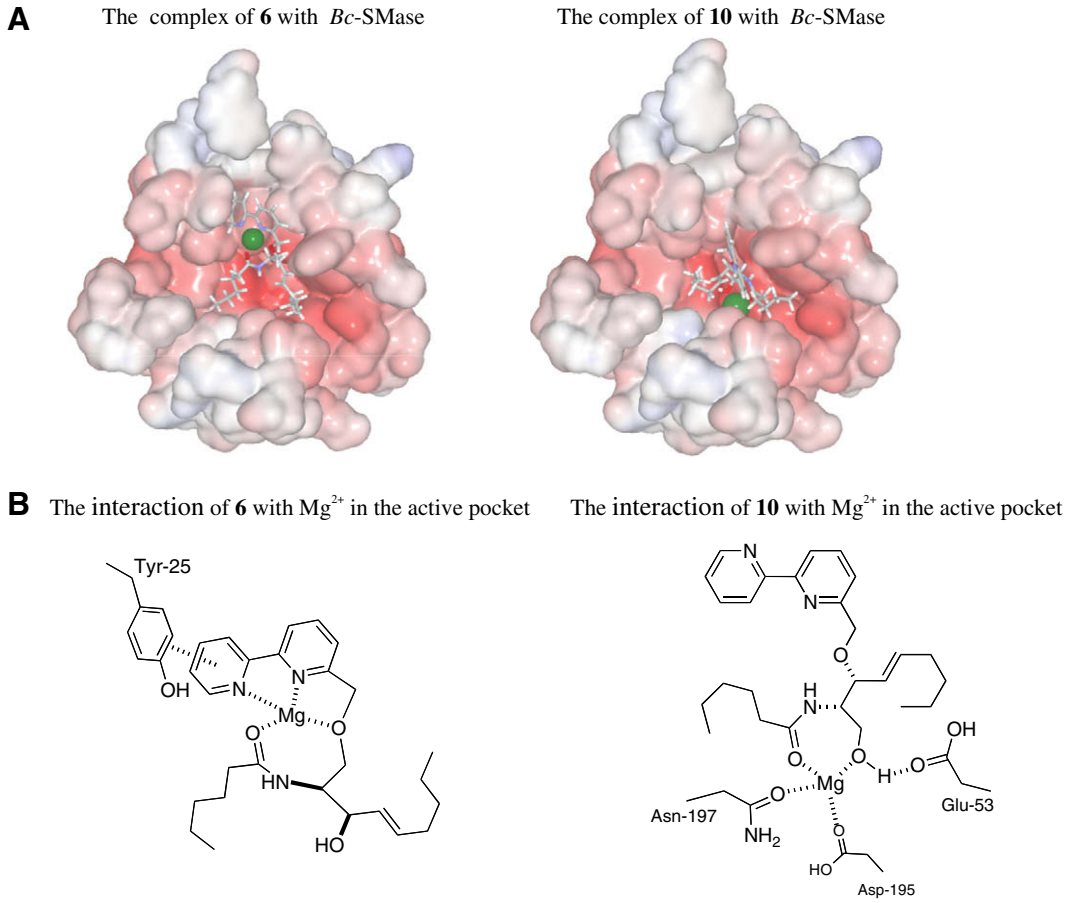


Figure 6. Simulation analysis of the docking of RY221B-a (**6**) and RY221B-b (**10**) for *Bc*-SMase. For simplification of calculations, the aliphatic side chains in **6** and **10** were truncated. (A) Simulation analysis of the docking of **6** and **10** for *Bc*-SMase. Left panel: docking between **6** and *Bc*-SMase. Right panel: docking between **10** and *Bc*-SMase. The most stable structure of **6** and **10** on the molecular electrostatic potential surface were shown. The red and blue surfaces show the negative and positive electrostatic potential surface, respectively. The gray, white, red, purple in the two compounds indicate carbon, hydrogen, oxygen, nitrogen, respectively. Mg^{2+} is represented in green. (B) The interactions between the *Bc*-SMase and **6** or **10** in the active pocket.

Table 1
Summary of docking energy measurement

	6	10
Docking energy (kCal/mol)	−117	−58

Lower numbers indicate more stable interactions.

SMase inhibitors yet identified (Fig. 4). In this assay, a notable difference in the inhibitory effect between **6** and **10** was also observed (Fig. 4). Lineweaver–Burk plot analysis revealed that the inhibitory effect of **6** is competitive with a K_i value of 5.2 μM, showing that the compound competed with sphingomyelin for binding to the catalytic site (Fig. 5).

Next, we investigated the mode of binding of **6** and **10** to the active pocket of *Bc*-SMase. Thus, we performed in silico docking simulation analyses of this interaction at the tertiary structure level using Molecular Operating Environment (MOE) software. Our results showed that **6** almost perfectly fit into the active pocket of *Bc*-SMase, moreover, the two nitrogen atoms of bipyridyl moiety, carbonyl oxygen of the amide and ethereal oxygen in **6** coordinate with Mg^{2+} in *Bc*-SMase (Fig. 6 A and B, left). By contrast, binding of **10** to the active pocket of *Bc*-SMase appears to be unstable (Fig. 6A, right). Moreover, the bipyridyl moiety in **10** was not chelated with Mg^{2+} in *Bc*-SMase (Fig. 6B, right). The structural difference between these two compounds is the position of the bipyridyl group, suggesting the importance of this moiety for binding to the *Bc*-SMase. Calculation of the binding energy using docking simulations revealed higher values for **6** compared with **10** (Table 1), further highlighting the importance of the position of bipyridyl moiety for more stable binding to *Bc*-SMase.

In conclusion, we created a novel and potent *Bc*-SMase inhibitor, **6** by molecular design based upon the phosphate-ligand analog hypothesis. The simulated binding of **6** with *Bc*-SMase supported the rationality of the molecular design process. We are currently attempting to prepare more hydrolase inhibitors by an extension of this approach.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bmcl.2010.05.042.

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