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Biological Evaluation of Sphingomyelin Analogues as Inhibitors of Sphingomyelinase

Minoru Taguchi,[a,*] Ken-ichi Goda,[b] Kikuo Sugimoto,[b] Tomoko Akama,[a] Kyoko Yamamoto,[a] Taizo Suzuki,[a] Yasumitsu Tomishima,[a] Mariko Nishiguchi,[a] Koshi Arai,[a] Kenzo Takahashi[a] and Takeo Kobori[b]

[a]*Medicinal Research Laboratories, Taisho Pharmaceutical Co., Ltd., 1-403 Yoshino-cho, Kita-ku, Saitama-shi, Saitama 331-9530, Japan*

[b]*Sagami Chemical Research Center, 2743-1 Hayakawa, Ayase-shi, Kanagawa 252-1193, Japan*

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Abstract—Seeking neutral sphingomyelinase inhibitors, we designed and synthesized hydrolytically stable analogues of sphingomyelin. These novel analogues replace the phosphodiester moiety of sphingomyelin with carbamate and urea moiety, resulting in inhibition of neutral sphingomyelinase. Compound **1** prevented ceramide generation and apoptotic neuronal cell death in a model of ischemia based on organotypic hippocampal slice cultures.

Introduction

Sphingomyelinase (SMase), which generates ceramide and phosphorylcholine by hydrolyzing the phosphodiester linkage of sphingomyelin (SM), is activated by stress conditions such as ischemia or cytokine stimulation, including tumor necrosis factor α (TNF-α) and interleukin 1β (IL-1β).[1] It has become apparent that no less than cell proliferation or cell differentiation, ceramide, a SMase product, plays an important role in the signal transduction of extracellular stimuli to apoptotic cell death as a lipid second messenger.[2] On the other hand, recent studies suggest the deep implication of apoptosis in stroke-related neuronal cell death and neurodegenerative diseases.[3] Thus, ceramide may play a key role in these neuronal diseases, in which case the inhibition of SMase may emerge as an unique therapy. The administration of exogenous ceramides induces apoptotic neuronal cell death,[4] while SMase inhibition suppresses apoptotic cell death in neuronal PC12 cells.[5,6] Various SMase activities have been noted in mammalian tissues and cells, including lysosomal acidic SMase (A-SMase), the membrane neutral magnesium-dependent SMase (N-SMase), the cytosolic magnesium-independent SMase, and so on. While it remains unclear which specific SMase activity contributes most significantly to neuronal apoptosis, N-SMase is distributed primarily in the brain. Ceramide generated by activation of N-SMase mediates hypoxic cell death in neural PC12 cells.[6] Thus, N-SMase may be a major factor in the pathogenesis of stroke and neurodegenerative disease, making the inhibition of N-SMase a topic worthy of further attention. We sought out selective N-SMase inhibitors that presented potential for use in the treatment of these diseases.

Previously, we reported that a non-phosphate analogue **1** that replaces the phosphodiester moiety of SM with a carbamate moiety selectively inhibited N-SMase.[7] Subsequently, we synthesized various new derivatives of **1** and examined the anti-apoptotic effects of **1** on neuronal cell death. In this paper, we describe herein structure activity relationships of derivatives of **1** and an evaluation of the bioactivity of **1** (Fig. 1).

Preparation of compounds

The urea analogue **7** was synthesized according to Scheme 1. The alcohol **3** prepared from 2-*N*-Boc-protected D-*erythro*-sphingosine **2** in five steps, as reported in our previous paper,[7] was oxidized to give **4**. The

*Corresponding author. Tel.: +81-4866-93029; fax: +81-4865-27254; e-mail: minoru.taguchi@po.rd.taisho.co.jp

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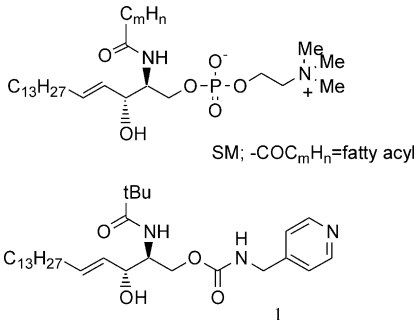
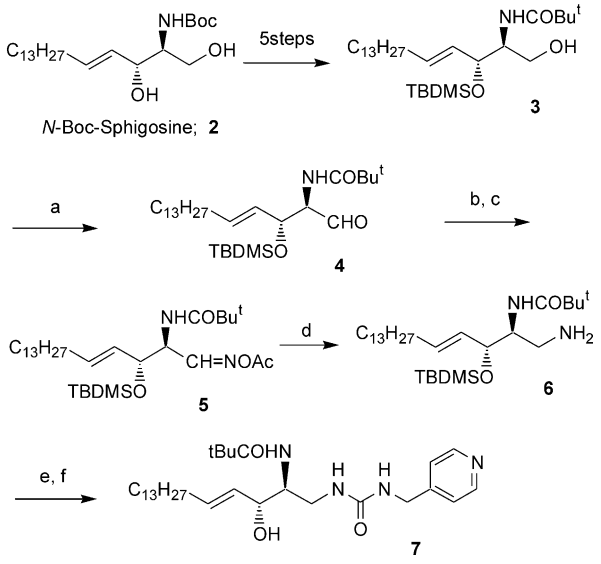


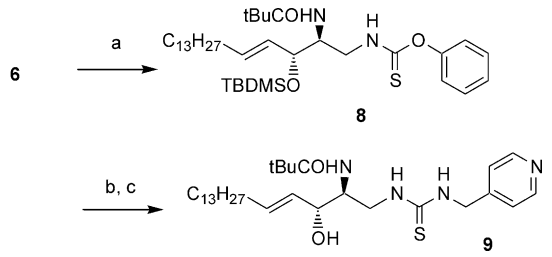
Figure 1. Sphingomyelin (SM) and sphingomyelinase (SMase) inhibitor **1**.



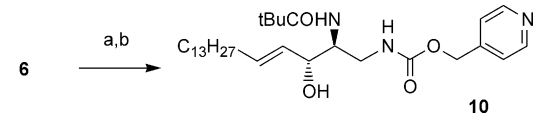
Scheme 1. (a) $PySO_3$, Et_3N, DMSO, THF, rt (90%); (b) $HONH_2$/HCl, *i*-Pr_2NEt, THF, rt (98%); (c) Ac_2O, Py, THF, 0 °C (74%); (d) $NaBH_4$, EtOH, −20 °C (58%); (e) Boc_2O, DMAP, CH_2Cl_2 then 4-$PyCH_2NH_2$, rt (74%); (f) Bu_4NF, THF, 0 °C (47%).

aldehyde **4** was treated with hydroxylamine, followed by acylation of the oxime to give the acetoxyimine **5**. Reduction of **5** with sodium borohydride gave the amine **6**. *t*-Butoxy carbonylation of the amine **6**, the resulting compound was treated with 4-pyridinemethylamine to give the protected urea derivative of **7**. Subsequent desilylation afforded the desired urea analogue **7**.

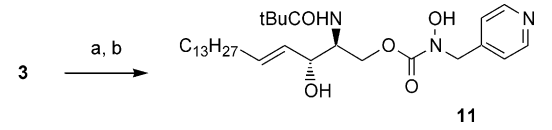
The thiourea analogue **9**, the carbamate analogue **10,** and the *N*-hydroxycarbamate analogue **11** were prepared as shown in Schemes 2–4, respectively. Thionocarbonylation of the amine **6** with phenyl chlorothionoformate gave the adduct **8**. Treatment of **8** with 4-pyridylmethylamine, followed by desilylation gave the desired thiourea **9**. The carbamate analogue **10** was prepared from **6**. The amine **6** was treated with trichloromethyl chloroformate, followed by condensation with 4-pyridylmethanol. Subsequent desilylation gave the desired carbamate analogue **10**. The *N*-hydroxycarbamate analogue **11** was prepared in a manner similar to that of **1.** The alcohol **3** was treated with trichloromethyl chloroformate, followed by condensation with (4-pyridylmethyl)hydoxylamine *O*-TBDMS to



Scheme 2. (a) ClC(S)OPh, Py, THF, −78 to −20 °C (33%); (b) $PyCH_2NH_2$, DMSO, rt (69%); (c) Bu_4NF, THF, 0 °C (73%).



Scheme 3. (a) $ClCO_2CCl_3$, Py, CH_2Cl_2, −78 to −20 °C, then $PyCH_2OH$, −20 °C (59%); (b) Bu_4NF, THF, 0 °C (72%).



Scheme 4. (a) $ClCO_2CCl_3$, DMAP, CH_2Cl_2, −78 to −20 °C, then $PyCH_2NHOTBDMS$, −20 °C (5%); (b) Bu_4NF, THF, 0 °C (87%).

give the protected derivative of **11**, which was desilylated to give the desired analogue **11**.

Compound **12** or **13**, an aliphatic side chain modification of **1**, was synthesized in a manner similar to that of **1**, in which an acetylene derivative corresponding to the side chain of **12** or **13** was used as a starting material. The saturated compound **14** or **15** was prepared from **1** or **12**, respectively, by catalytic reduction (H_2/10% Pd/C/MeOH).

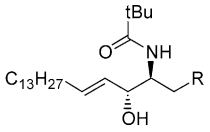
Biological effects

The inhibitory activity of synthesized novel analogues on N-SMase was examined using rat brain microsomes, previously described.[7] The results are given in Tables 1 and 2. The inhibitory activity of the urea **7** (IC_{50} = 2.9 μM) was almost equal to that of carbamate **1**. The thiourea **9**, the reverse carbamate **10**, and the *N*-hydroxy carbamate **11** were 10–20 times less potent than compound **1**.

On the other hand, compounds **12** or **13,** which have different carbon chains, showed somewhat weaker inhibitory activity than compound **1**. Saturated compounds **14** and **15** decreased the inhibitory activity.

For a functional evaluation of compound **1**, we used a model of ischemia based on organotypic hippocampal slice cultures. Organotypic hippocampal slice cultures were prepared from 7-day-old rats, as described by Sakaguchi et al.,[8] but with certain modifications. The hippocampal slices were cultured for 7 days, then exposed to ischemic stress with oxygen-glucose deprivation for 24 h, followed by reperfusion for 24 h. Apoptotic cell death was determined in each slice by the

Table 1. Inhibitor potential of the derivatives of **1**



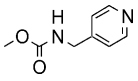
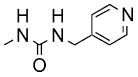
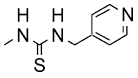
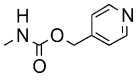
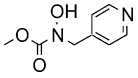
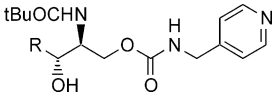
Compd	R	IC_{50} μM
1		2.8
7		2.9
9		45
10		58
11		21

Table 2. Inhibitor potential of the derivatives of **1**



Compd	R	IC_{50} μM
1	$C_{13}H_{27}$	2.8
12	$C_{10}H_{21}$	6.8
13	$C_{15}H_{31}$	6.6
14	$C_{13}H_{27}$	> 100
15	$C_{10}H_{21}$	> 100

terminal deoxynucleotidyl transferase-mediated dUTP-biotin nick-end labeling (TUNEL) method, a histochemical assay of DNA fragmentation.[9] A basal level of neuronal cell death due to apoptosis occurred under these culture conditions. Following ischemic stress, the cultured hippocampal slices contained a large number of TUNEL-positive neurons; 3 μM of compound **1** significantly prevented this increase in apoptotic cell death (Fig. 2).

We also examined ceramide levels in hippocampal slices exposed to ischemic stress with and without compound **1**. As shown in Figure 3, ceramide levels were increased by ischemia. This increase was significantly attenuated by compound **1**, suggesting that compound **1** protects neurons from apoptosis by inhibiting ceramide accumulation, which has been reported to induce apoptosis.

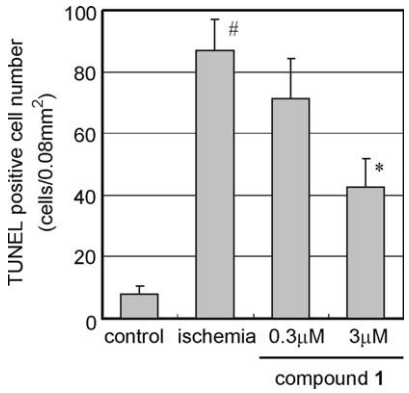


Figure 2. Effect of **1** on organotypic hippocampal slice after ischemia. Results are means of nine different experiments. The SE is given. #$p < 0.05$, significantly different from control (Student's *t*-test). *$p < 0.05$, significantly different from ischemia (Dunnett's multiple comparison test).

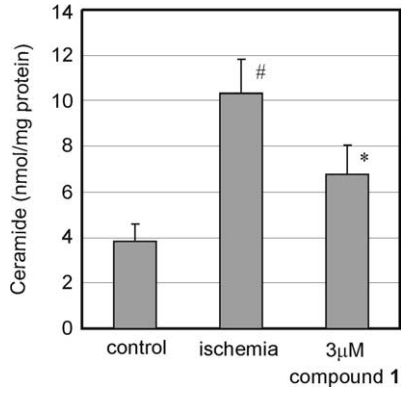


Figure 3. Compound **1** inhibits ceramide accumulation induced by in vitro ischemia. Results are means of four different experiments. The SE is given. # $p < 0.05$, significantly different from control (Student's *t*-test). *$p < 0.05$, significantly different from ischemia (Student's *t*-test).

The results of this study appear to confirm that the selective N-SMase inhibitor **1** attenuates neuronal cell death and ceramide generation induced by ischemia. These results suggest that N-SMase inhibitors should be valuable in the treatment of stroke and other neurodegenerative diseases. The paper also proposes various new surrogates for the phosphodiester moiety of SM and demonstrates the possibility of conversion to low molecular weight and non-phosphate compounds. Based on these results, we may look forward to progress toward the development of SMase inhibitors.

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