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Sphingomyelin Analogues as Inhibitors of Sphingomyelinase

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Abstract—To search for neutral sphingomyelinase inhibitors we designed and synthesized hydrolytically stable analogues of sphingomyelin. The novel compounds **8** and **9** which were replaced the phosphodiester moiety of sphingomyelin with the carbamate moiety showed inhibitory activity with an IC_{50} value of μM on neutral sphingomyelinase in rat brain microsomes. Compound **8i** showed a selective neutral sphingomyelinase inhibitory activity.

Introduction

Recent studies suggested that apoptosis is involved in neuronal cell death which accompanies stroke and neurodegenerative diseases.[1] Additionally, it was suggested that ceramide, an intracellular lipid messenger, induces apoptotic neuronal cell death.[2] Ceramide is the product of sphingomyelinase (SMase)-catalysed hydrolysis of sphingomyelin (SM), one of the most abundant sphingolipid species in plasma membranes of cells in mammalians (Fig. 1).

Stress conditions such as ischemia, stimuli by cytokines or irradiation,[3] activate SMase, increase the amount of ceramide and can initiate apoptosis.[2] Many different SMase activities have been noted in mammalians, including lysosomal acidic SMase (A-SMase),[4] the membrane neutral magnesium-dependent SMase (N-SMase),[5] the cytosolic magnesium-independent SMase,[6] and others. Although the relevance of each activity on specific cellular reactions and diseases has not been clarified, N-SMase distributes mainly in the brain[7] and may be the major activity participating in the pathogenesis of stroke and neurodegenerative diseases. It was reported that ceramide generated by activation of N-SMase mediated hypoxic cell death in neuronal PC12 cells.[8] Therefore the inhibition of N-SMase warrants study as a possible therapy for subjects with the neuronal diseases. Additionally, selective N-SMase inhibitors will be useful to clarify the physiological functions of N-SMase.

Nara et al. reported that Scyphostatin, a constituent of *Dasyscyphus mollissimus* inhibits N-SMase of rat brain microsomes with an IC_{50} value of 1 μM[9] and Omura et al. reported N-SMase inhibitors of microbial origin, KF-1040A ($IC_{50} = 5.4$ μM)[10] and Alutenusin ($IC_{50} = 40$μM).[11] On the other hand, Yokomatsu et al. reported a SM difluoromethylene analogue which inhibited N-SMase of bovine brain microsomes with an IC_{50} value of 3.3 μM[12] and Katsumura et al. reported SM carbon analogues.[13]

We have searched for N-SMase inhibitors among the analogues of SM. In designing the analogues, we considered simplification of synthesis and improvement of the poor solubility of SM and we converted the phosphodiester moiety the hydrolysis position in SMase. For improvement of the solubility, we studied shortening of the 2-*N*-acyl group and replacement of the trimethylammonium group with the dialkylamino or pyridyl group which was recognized as the surrogate for the choline moiety in investigation of the muscarinic antagonist. We also studied ester or carbamate analogues for conversion of the phosphodiester moiety. Therefore we found that novel carbamate compound, as

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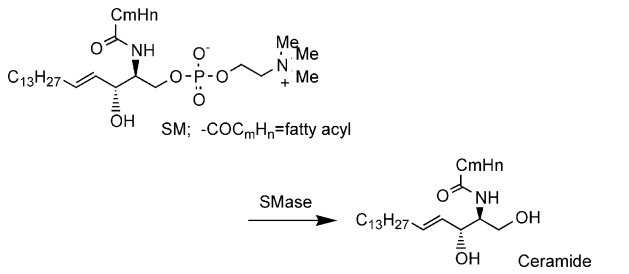
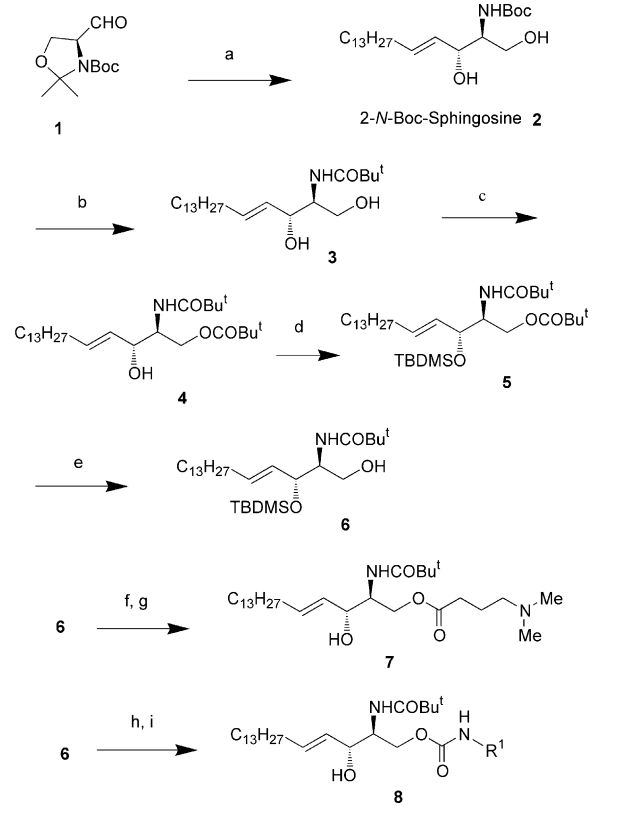


Figure 1. Metabolism of Sphingomyelin (SM) by Sphingomyelinase (SMase).

analogues of SM, showed a selective inhibitory activity on N-SMase. We describe here the syntheses and structure activity relationships of N-SMase inhibitors we investigated.

Preparation of Compounds

The synthetic method we used is shown Scheme 1. The desired intermediate, 2-*N*-Boc-protected D-*erythro*-sphingosine **2** $[\alpha]_D^{20}$ −1.1 (*c* 1.1,$CHCl_3$); lit.$[\alpha]_D^{25}$ −1.4 (*c* 1.1, $CHCl_3$), was synthesized from the Garner aldehyde **1** according to the synthesis method of Herold et al.[14] The ester analogue **7** and the carbamate analogues **8** were obtained by introducing the desired substitution into the 1-hydroxy group of **2**. Thus the 2-*N*-Boc group of **2** was deprotected under acidic conditions and *N*-acylation with pivaloyl chloride yielded 2-*N*-pivaloyl sphingosine **3**. Pivaloylation of the 1-hydroxyl group of **3**, followed by silylation of the 3-hydroxyl group with *t*-butyldimethylsilyl chloride, yielded **5**. Deprotection of the 1-pivaloyloxy group of **5** with DBU gave 2-*N*-pivaloyl-3-*O*-*t*-butyldimethylsilyl-D-*erythro*-sphingosine **6** that was condensed with 4-(dimethylamino)butyric acid. Subsequent desilylation afforded the desired ester **7**. On the other hand, **6** was treated with trichloromethyl chloroformate and the resulting compound was reacted with amines corresponding to the analogues **8**. Subsequently desilylation gave the desired carbamate analogues **8**. In transformation of the 2-*N*-acyl group, the 2-amino group of sphingosine was coupled with carboxy acids and the resulting compounds were converted to the 2-*N*-isobutyryl analogue **9**, the 2-*N*-acetyl analogue **10** and the 2-*N*-steroyl analogue **11**, respectively, using the method described above.

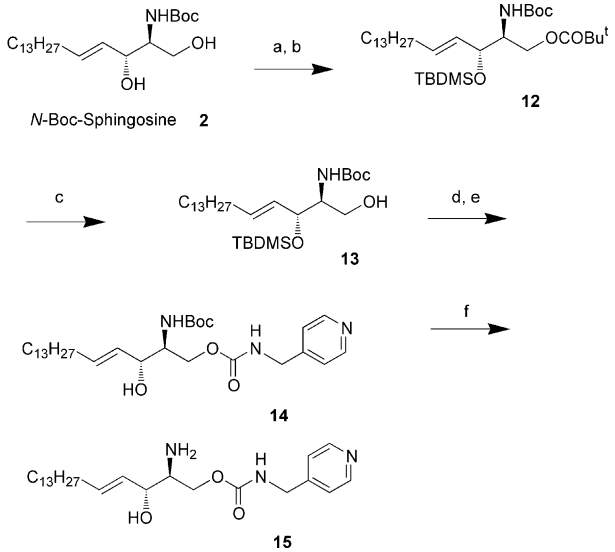


Scheme 1. (a) Lit. 14; (b) TFA, 0 °C then *t*BuCOCl, Et_3N, THF, rt (50%); (c) *t*BuCOCl, Py, −10 °C (82%); (d) TBDMSCl, imidazole, DMF, rt (95%); (e) DBU, MeOH, rt (97%); (f) $Me_2N(CH_2)_3CO_2H$, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide, DMAP, $CHCl_3$, rt (80%); (g) 2% HF, Py, CH_3CN, rt (42%); (h) $ClCO_2CCl_3$, Py, CH_2Cl_2, −78 °C to −15 °C, then R^1NH_2, −30 °C (40–90%); (i) 2% HF, Py, CH_3CN, rt (59–97%).

The 2-*N*-Boc-1-carbamate analogue **14** was prepared in the following manner, as shown in Scheme 2. The 1-hydroxyl group of 2-*N*-Boc sphingosine **2** protected by the pivaloyl group was followed by *t*-butyldimethylsilylation of the 3-hydroxyl group, gave **12**. Hydrolysis of the 1-pivaloyl group of **12**, the resulting alcohol **13** was treated in a manner similar to that described for the preparation of **8** from **6**, to give the desired carbamate analogue **14**. Furthermore, the 2-amino-1-carbamate analogue **15** was obtained by deprotection of the 2-*N*-Boc on **14**.

Biological Effects

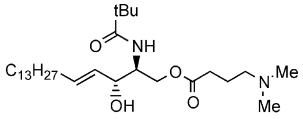
The inhibitory activity of our synthesized analogues on N-SMase was examined using rat brain microsomes as the enzyme source. To measure N-SMase activity,



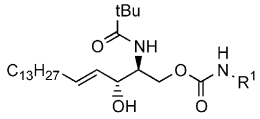
Scheme 2. (a) *t*BuCOCl, Py, −20 °C (91%); (b) TBDMSCl, imidazole, DMF, rt (quant); (c) DBU, MeOH, rt (93%); (d) $ClCO_2CCl_3$, Py, CH_2Cl_2, −78 °C to −15 °C, then 4-$PyCH_2NH_2$, −30 °C (63%); (e) Bu_4NF, THF, 0 °C (95%); (f) TFA, 0 °C (quant).

microsomes (50μg of protein) was incubated for 25 min at 37 °C in 80μL of buffer containing 0.3mM ^{14}C-labelled SM, 100 mM Tris–HCl (pH 7.5), 20 mM $MgCl_2$, 0.25% TritonX-100 and various concentrations of test compounds. Radioactive phosphorylcholine produced from ^{14}C-labelled SM was extracted with chloroform/methanol (2/1,v/v). The amount of phosphorylcholine in the aqueous phase was determined by scintillation counting. Results are shown in Tables 1 and 2. The IC_{50} value of compound **7** transformed to an ester moiety with dimethylaminobutylate was 31μM. This transformation of the ester moiety to the carbamate moiety increased the inhibitory activity to 4.1μM (compound **8a**). Transformation from the methyl group of the amine moiety to the bulky *i*-propyl group led to a 4-fold less potent activity (compound **8d**). Replacement of a nitrogen atom of the amino group with a carbon atom resulted in a significant loss of the inhibitory activity (compound **8e**). Compounds that were replaced the amino group with the pyridyl group were somewhat more potent than compound **8a**. In the 2-pyridyl **8g**, 3-pyridyl **8h** or 4-pyridyl analogue **8i**,[15] the activity was almost the same (IC_{50} = 2 to 5μM). The inhibitory activity of compounds **8h**, **8i** was almost equal compared with Scyphostatin.[9] Regarding the distance between carbamate linkage and the pyridyl group, the one-carbon tether was most active and a direct bond and the two-carbon tether were less active. Activity of the long 2-*N*-fatty acyl compound, the 2-*N*-steroyl analogue **11** was not found, perhaps solubility was low. The 2-*N*-isobutyryl analogue **9** showed activity (IC_{50} = 1.8 μM) almost equivalent to the 2-*N*-pivaloyl analogue **8i**. The short 2-*N*-acyl chain compound, the 2-*N*-acetyl analogue **10**, was less active. On the other hand, the 2-*N*-Boc-1-carbamate analogue **14** and the 2-amino-1-carbamate analogue **15** did not show activity.

Table 1. Inhibitor capacity of ester and carbamate analogues



7 IC_{50}=31μM



8a-k

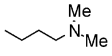
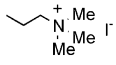
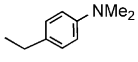
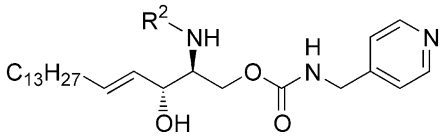
Compd	R^1	IC_{50} μM
8a		4.1
8b		4.9
8c		8.7
8d		18
8e		77
8f		7.9
8g		5.0
8h		2.1
8i		2.8
8j		8.4
8k		8.2

Table 2. Inhibitor capacity of carbamate analogues



Compd	R^2	IC_{50} μM
8i	$COBu^t$	2.8
9	$COPr^i$	1.8
10	COMe	>100
11	$COC_{17}H_{35}$	>100
14	CO_2Bu^t	>100
15	H	>100

To examine the specificity of compound **8i** for N-SMase, we investigated effects of this compound against A-SMase from rat brain lysosomes, bacterial SMase (Bacillus cereus SMase; Sigma, S-9396) and phospholipase C. No significant inhibition of each lipase occurred at 10 and 100 μM. Additionally, we found no significant activity, that is, IC_{50} > 10μM, of **8i** on 29 receptors and 10 enzymes (data not shown). These results are interpreted to mean that **8i** is a selective inhibitor of N-SMase.

From this study, we confirmed that the carbamate analogues are selective N-SMase inhibitors. The carbamate moiety is available as a surrogate for the phosphodiester moiety of SM, which is not hydrolyzed by SMase. Additionally, the 2-*N*-pivaloyl or 2-*N*-isobutyryl analogues are more advantageous in comparison with long 2-*N*-fatty acyl compounds with regard to solubility during synthesis and for evaluation of biological activity. We are examining the possibility of using these novel N-SMase inhibitors to treat subjects with ceramide-induced cell death.

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15. Spectral data for compound **8i**: $[\alpha]_D^{20}$ −1.3(c 1.1,$CHCl_3$); ^1H NMR($CDCl_3$, 200MHz) δ 0.88(t, J=6.5 Hz, 3H), 1.15 (s, 9H), 1.20–1.44 (m, 22H), 2.02 (m, 2H), 3.72 (m, 1H), 4.13–4.17 (m, 3H), 4.34–4.48 (m, 3H), 5.45 (dd, J=6.6, 15.4 Hz, 1H), 5.74 (dt, J=15.3, 6.6 Hz, 1H), 6.29 (d, J=7.4 Hz, 1H), 7.20 (m, 2H), 8.57 (m, 2H); IR (KBr): 3320, 2959, 2919, 2852, 1705, 1635, 1537, 1471, 1420, 1368, 1271, 1154, 1095, 1043, 1007, 971, 905, 796, 718cm^{-1}; MS(SIMS) *m*/*e*: 518 (MH^+); HRMS(ESI) *m*/*e*: calcd for $C_{30}H_{51}N_3O_4$: 518.3958 (MH^+). Found: 518.3963.