Pergamon

Bioorganic & Medicinal Chemistry Letters 11 (2001) 1277–1280

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Synthesis and Evaluation of a Difluoromethylene Analogue of Sphingomyelin as an Inhibitor of Sphingomyelinase

Tsutomu Yokomatsu,[a,*] Hiroaki Takechi,[a] Takeshi Akiyama,[a] Shiroishi Shibuya,[a] Takaaki Kominato,[b] Shinji Soeda[b] and Hiroshi Shimeno[b,*]

[a]*School of Pharmacy, Tokyo University of Pharmacy & Life Science, 1432-1 Horinouchi, Hachioji, Tokyo 192-0392, Japan*
[b]*Faculty of Pharmaceutical Sciences, Fukuoka University, 8-19-1 Nanakuma, Jonan-ku, Fukuoka 814-0180, Japan*

Received 23 January 2001; accepted 10 March 2001

Abstract—A sphingomyelin analogue **2**, in which the long alkenyl chain and the phosphodiester moiety of sphingomyelin were replaced by a phenyl and an isosteric difluoromethylenephosphonic acid, was prepared to evaluate its inhibitory potency to sphingomyelinase. The analogue non-competitively inhibited the neutral sphingomyelinase in bovine brain microsomes with an IC_{50} of 400 μM. The compound had the ability to suppress tumor necrosis factor α-induced apoptosis of PC-12 neurons at a low concentration of 0.1 μM.

Sphingomyelinase (SMase) specifically catalyzes the hydrolysis of the phosphoester linkage of membrane sphingomyelin (SM) to generate ceramide and phosphorylcholine. There are several isoforms, distinguished by different pH optima, cellular topology, and cation dependence. An Mg^{2+}-dependent neutral (N-) SMase mainly operates at plasmamembranes,[1] while acidic (A-) SMase is localized in endosomal/lysosomal compartments.[2] Further, Mg^{2+}-independent N-SMase[3] and alkaline SMase[4] are found in cytosol and in the gastrointestinal tract, respectively. Activation of N-SMase and/or A-SMase in cells occurs in response to growth factors, cytokines, chemotherapeutic agents, and stress conditions. The generated ceramide is believed to play a pivotal role in regulation of cell growth and differentiation, cell cycle arrest, apoptosis, and infammation as the lipid messenger.[5] However, direct links between SMases and specific signaling systems have not yet been established. To establish a clear picture of the metabolic links, the synthesis of specific SMase inhibitors is strongly required. Additionally, the SMase inhibitor is expected to have some clinical values, because ceramide generation following SM hydrolysis might be implicated in pathological states such as AIDS.[6]

In a search for a novel structural class of SMase inhibitors, we have been interested in the synthesis and biological evaluation of a hydrololytically stable analogue **1** of SM, in which the oxygen of the phosphoester linkage is replaced by a difluoromethylene (CF_2) unit (Fig. 1).

The particular utility of replacement of a phosphoric ester oxygen of *p*-Tyr in a peptidyl framework with a CF_2-unit has been demonstrated in the synthesis of useful biochemical tools for studying the mechanism of tyrosine-kinase-mediated signal transductions,[7,8] since the resulting phosphonate analogue mimics accurately the parental phosphate in its isosterical and isopolar properties.[9] However, the strategy has not been applied to synthesis of a hydrolytically stable analogue of SM, which might be useful to create SMase inhibitors valuable for studying the ceramide-mediated signaling cascade.[10]

In a modification of sphingolipids, shortening the sphingoid backbone as well as introducing an aromatic group in the backbone are recently proved to be a useful strategy to create analogues having increased solubility and cell permeability which are available for studies in vitro and in vivo.[11] Recent studies on initial velocities of hydrolysis of short-chain analogues of SM by SMase

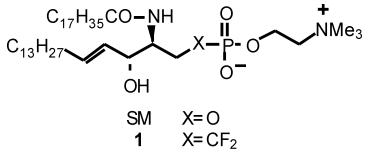
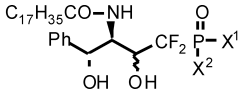


Figure 1. Sphingomyelin (SM) and its difluoromethylene analogue.

*Corresponding authors. Tel.: +81-426-76-3251; fax: +81-426-76-3239; e-mail: yokomatu@ps.toyaku.ac.jp (T. Yokomatsu); tel.: +81-92-871-6631; fax: +81-92-863-0389; e-mail: shimeno@fukuoka-u.ac.jp (H. Shimeno)

0960-894X/01/$ - see front matter
PII: S0960-894X(01)00179-2

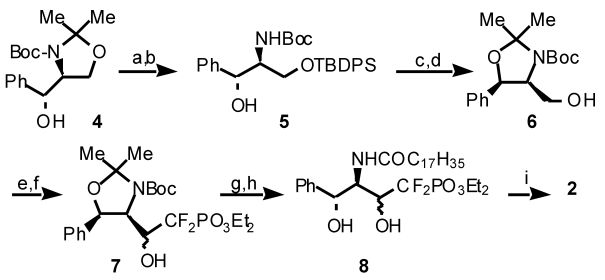
reveal that the length of the alkenyl side chain is not important but the D-*erythro* stereochemisty is critical for interaction between the substrates and the enzyme.[10] Considering these findings, we decided to synthesize and evaluate the short-chain analogue **3** and the des coline derivative **2**, prior to the synthesis of **1**. An additional feature of our interest in the analogues is to install a hydroxyl functionality to the α position of the CF_2-unit. A framework that is composed of proximate hydroxyl and phosphonate functionalities is known to have the strong chelating ability of metals.[12] Therefore, the analogue **3** might favorably interact with SMase because of the Mg^{2+}-dependency. In this paper, we describe the synthesis of **2** and its inhibitory potency against various types of SMases. The results show that the coline moiety is not necessary to inhibit SMase. Preliminary data on the anti-apoptotic effect of **2** on the cell death of PC-12 neurons are also presented.



2 $X^1=X^2=OH$
3 $X^1=OCH_2CH_2N^+Me_3$; $X^2=O^-$

Results and Discussion

The analogue **2** was synthesized from the known Garner aldehyde[13] (*tert*-butyl (*S*)-4-formyl-2,2-dimethyl-3-oxazolidine-1-carboxylate) as shown in Scheme 1. According to the literature procedures,[14] the Garner aldehyde was stereoselectively transformed to the alcohol **4**. Deprotection of the acetonide, followed by selective silylation with *tert*-butyldiphenylsilyl chloride (TBDPS-Cl), gave **5**, $[\alpha]_D^{25}$ +6.03 (*c* 0.9, $CHCl_3$), in 88% yield for two steps. *N*,*O*-Acetalization of **5**, followed by desilylation gave **6**, $[\alpha]_D^{25}$ −29.0 (*c* 0.9, $CHCl_3$), in 81% yield. The alcohol **6** was oxidized with the Dess–Martin periodinane;[15] the resulting aldehyde was treated with lithium salts of diethyl difluoromethylphosphonate at −78 °C in THF to give the adduct **7** as an inseparable mixture (1:1) of diastereoisomers in 60% yield.[16] The acetonide and *N*-Boc of **6** were removed and the resulting amino alcohol was acylated with palmitoyl chloride to give **8** in 60% yield. Deprotection of diethyl ester **8** under the conventional conditions gave **2** (an amorphous powder) in virtually quantitative yield as a mixture (1:1) of diastereoisomers. Attempted separation of the diastereoisomers failed.[17]



Scheme 1. Reagent: (a) *p*-TsOH (cat.), MeOH, 25 °C, 7.5 h; (b) TBDPSCl, imidazole, DMF; (c) 2,2-dimethoxypropane, *p*-TsOH (cat.), benzene, 80 °C; (d) Bu_4NF, THF; (e) Dess–Martin periodinane, CH_2Cl_2; (f) $LiCF_2PO_3Et_2$, THF, −78 °C; (g) 3 M HCl, EtOAc; (h) palmitoyl chloride, Et_3N, DMAP (cat.); (i) TMSBr, CH_2Cl_2, followed by MeOH.

Nara et al.[18,19] recently reported an N-SMase inhibitor, scyphostatin, from a discomycete, *Trichopeziza mollissima*, where they used rat brain microsomes (Ms) as the enzyme source. We chose Ms from bovine organs as the enzyme source. Prior to the inhibition experiments with **2**, we examined the Mg^{2+}-dependency of N-SMases isolated from the the liver and brain Ms, respectively, since the Mg^{2+}-dependent N-SMases are known to be critical to induce apoptosis of cells.[5] As shown in Figure 2, the activity of N-SMase in the brain Ms was Mg^{2+}-dependent, while the Mg^{2+}-dependency of N-SMase in the liver Ms was very weak.[20] Figure 3 shows the concentration-dependent inhibition of N-SMases by **2** which was more potent against N-SMase present in brain Ms than that in liver Ms. IC_{50} value of **2** for the brain N-SMase was found to be approx. 400 μM. The obtained IC_{50} value is relatively high in comparison with that of scyphostatin for rat brain N-SMase (IC_{50} = 1.0 μM).[19] Recently, Liu and Hannun demonstrated that, in human leukemia cells, glutathione (GSH) blocked the activity of N-SMase at the physiological concentration range of 1–20 mM.[21] Lineweaver–Burk plot analysis indicates that GSH and oxidized GSH (GSSG) are mixed-type inhibitors against N-SMase in bovine brain Ms (Fig. 4A). The kinetic analysis of **2** also revealed it to be a non-competitive type of inhibitor against the N-SMase (Fig. 4B).

We next examined the specificity of **2** for SMases obtained from various origins and for ceramide synthase present in bovine liver Ms (Table 1). As shown

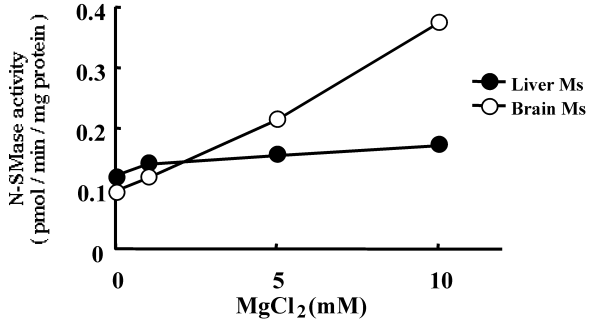


Figure 2. Magnesium-dependency of N-SMase activity in bovine liver and brain Ms.

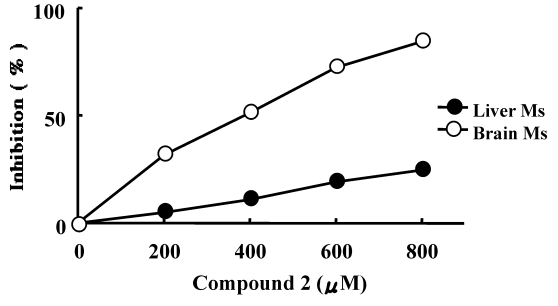


Figure 3. Determinatoin of inhibitory potency of **2** against N-SMase activity in bovine liver and brain Ms.

in Figure 3, the compound **2** at 400 μM inhibited 50% of the N-SMase activity in bovine brain Ms, but had a weak inhibitory effect on N-SMase in the liver Ms (15% inhibition). On the other hand, the compound **2** had no effect on N-SMase derived from *Bacillus cereus* at 400 μM. Of the SMases tested, interestingly, **2** at 400 μM showed the most potent inhibitory effect on A-SMase present in bovine brain lysosomes (80% inhibition). The A-SMase gene gives rise to both lysosomal A-SMase and secretory N-SMase, which is Zn^{2+}-dependent.[22] Thus, because the protein nature and catalytic property of A-SMase are very similar to those of secrotory N-SMases, the specificity of **2** may not be limited. We also tested the inhibition of ceramide synthase present in bovine liver Ms, because the enzyme catalyzes the conjugation of acyl-CoA and sphingosine, which is the skeletal molecule of SM. The result indicates that **2** at 400 μM has no inhibitory effect on this enzyme.

Finally, we show preliminary data indicating that **2** prevents tumor necrosis factor (TNF)-α-induced cell death of PC-12 neurons[23] at a low concentration of 0.1 μM (Fig. 5). Panel A shows the intact PC-12 neurons (control) cultured for 24 h in serum-free medium. The cells treated for 24 h with 500 units/mL TNF-α appear round and show characteristics of necrotic and/or apoptotic cells (panel B). However, in combination with 0.1 μM of **2**, PC-12 neurons maintained the intact neuronal cell morphology for at least 24 h (panel C).

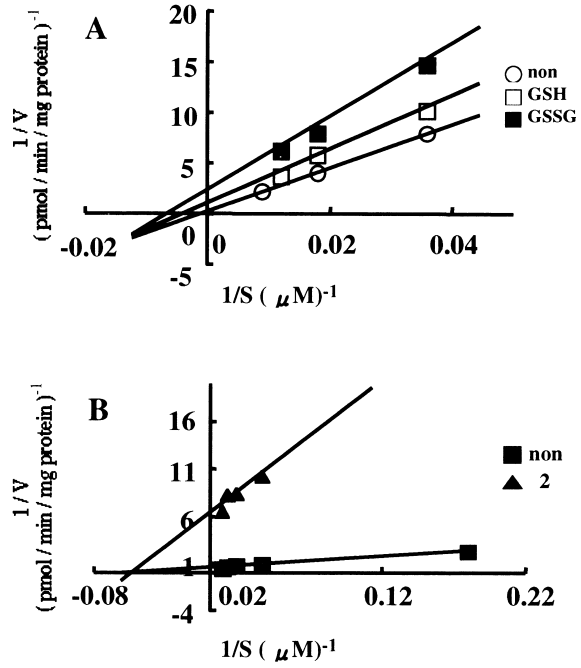


Figure 4. Lineweaver–Burk plot analyses of SM hydrolysis by bovine brain Ms in the presence of glutathiones (panel A) or **2** (panel B).

Table 1. Inhibitory potency of **2** at 400 μM (IC_{50} value for brain N-SMase) against SMases and ceramide synthase

Enzyme	Origin	Inhibition (%)
N-SMase	Bovine brain Ms	50
N-SMase	Bovine liver Ms	15
N-SMase	*Bacillus cereus*	0
A-SMase	Bovine brain lysosomes	80
Ceramide synthase	Bovine liver Ms	0

In summary, we have synthesized a difluoromethylene analogue of SM and shown that the compound **2** noncompetitively inhibits the Mg^{2+}-dependent N-SMase in bovine brain Ms with an IC_{50} value of 400 μM. However, scyphostatin from discomycete, *Trichpeziza mollissima* is more potent than **2** as an inhibitor of mammalian Mg^{2+}-dependent N-SMase.[19] Similarly to the properties of **2**, scyphostatin inhibits lysosomal A-SMase with IC_{50} value of 49.3 μM, but not N-SMases from *S. aureus* and *B. cereus*.[19] The inhibition of Mg^{2+}-dependent N-SMase by scyphostatin was mixed-type.[18] Nara et al.[19] have shown the utility of scyphostatin as an inhibitor of Mg^{2+}-dependent N-SMase by using carrageenin-induced paw edema in rats. During the inflammation processes, TNF-α, interleukin(IL)-1β and other cytokines play the role of central contributors. The cytokines such as TNF-α and IL-1β activate the Mg^{2+}-dependent N-SMase on inflammatory cells and generate the lipid messenger, ceramide.[24] Therefore, the 50% suppression of carrageenin-induced paw edema by po administration (100 mg/kg) of scyphostatin may be due to the inhibition of N-SMase. Nara et al. also showed that scyphostatin inhibits lipopolysaccharide-induced IL-1β production in human peripheral monocytes with IC_{50} value of 0.1 μM.[19]

In the present study, we show for the first time the possibility that the SMase inhibitor is also available for the suppression of neuronal cell death induced at least by TNF-α. The compound **2** morphologically inhibited TNF-α-induced cell death of PC-12[23] neurons at a concentration of 0.1 μM. We confirmed that the TNF-α-induced cell death was apoptotic and that the cytokine-induced activation of N-SMase on PC-12 neurons was significantly inhibited by the co-presence of **2** (these results will be described elsewhere). Recent studies suggest that neurons die from apoptosis in oxygen-deprived brains including stroke[25] and trauma[26] and also in the brains of Alzheimer's patients.[27] Such unscheduled apoptosis may largely contribute to the pathology of neurodegenerative diseases. Therefore, the prevention of ceramide-induced apoptosis by treatment with an

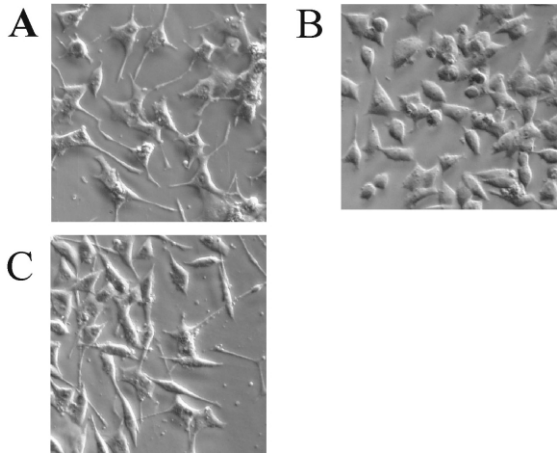


Figure 5. Effect of **2** on TNF-α-induced cell death of PC-12 neurons.

SMase inhibitor seems to be an attractive therapeutic strategy for these diseases. Further structural improvement of **2** and studies on their action related to neuronal cell death in vitro and in vivo are needed.

Acknowledgements

This work was supported in part by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science, Sports and Culture of the Japanese Government.

References and Notes

1. Hostetler, K. Y.; Yazaki, P. J. *J. Lipid Res*. **1979**, *20*, 20.
2. Ferlinz, K.; Hurwitz, R.; Vielhaber, G.; Suzuki, K.; Sandhoff, K. *Biochem. J*. **1994**, *301*, 855.
3. Okazaki, T.; Bielawska, A.; Domae, N.; Bell, R. M.; Hannun, Y. A. *J. Biol. Chem*. **1994**, *269*, 4070.
4. Nyberg, L.; Nilsson, A. *Biochim. Biophys. Acta* **1995**, *1259*, 49.
5. Levade, T.; Jaffrézou, J.-P. *Biochim. Biophys. Acta* **1999**, *1438*, 1.
6. Chatterjee, S. *Arterioscler. Thromb. Vasc. Biol*. **1998**, *18*, 1523.
7. (a) Burke, T. R., Jr.; Smyth, M. S.; Otaka, A.; Nomizu, M.; Roller, P. P.; Wolf, G.; Case, R.; Shoelson, S. E. *Biochemistry* **1994**, *33*, 6490. (b) Chen, L.; Wu, L.; Otaka, A.; Smyth, M. S.; Roller, P. P.; Burke, T. R.; den Hentog, J., Jr.; Zhang, Z.-Y. *Biochem. Biophys. Res. Commun*. **1995**, *216*, 976. (c) Yokomatsu, T.; Murano, T.; Umesue, I.; Soeda, S.; Shimeno, H.; Shibuya, S. *Bioorg. Med. Chem. Lett*. **1999**, *9*, 529 and the references cited therein.
8. A similar replacement strategy has been also applied to synthesis of a hydrolytically stable analogue of phospholipids as an inhibitor of phospholipase C from *Bacillus cereus*: Martin, S. F.; Wong, Y.-L.; Wagman, A. S. *J. Org. Chem*. **1994**, *59*, 4821.
9. (a) Chamber, R. D.; O'Hagan, D.; Lamount, R. B.; Jain, S. C. *J. Chem. Soc., Chem. Commun*. **1990**, 1053. (b) O'Hagan, D.; Rzepa, H. S. *J. Chem. Soc., Chem. Commun*. **1997**, 645 and the references cited therein.
10. (a) Synthesis and evaluation of methylene analogues of SM as an SMase inhibitor were recently described by Katsumura: Hakogi, T.; Monden, Y.; Iwama, S.; Katsumura, S. *Org. Lett*. **2000**, *2*, 2627. (b) Murakami, M.; Iwama, S.; Fujii, S.; Ikeda, K.; Katsumura, S. *Bioorg. Med. Chem. Lett*. **1997**, *7*, 1725.
11. Van Overmeire, H.; Boldin, S. A.; Dumont, F.; Van Calenbergh, S.; Slegers, G.; De Keukeleire, D.; Futerman, A. H.; Herdewijn, P. *J. Med. Chem*. **1999**, *42*, 2697.
12. Fracis, M. D.; Martodam, R. R. In *The Role of Phosphonates in Living Systems*; Hilderbrand, R. L., Ed.; CRC Press: Boca Raton, 1983; pp 57–96.
13. Garner, P.; Park, J. M. *J. Org. Chem*. **1987**, *52*, 2361.
14. Nishida, A.; Sorimachi, H.; Iwaida, M.; Matsumizu, M.; Kawate, T.; Nakagawa, M. *Synlett* **1998**, 389.
15. Dess, D. B.; Martin, J. C. *J. Am. Chem. Soc*. **1991**, *113*, 7277.
16. Martin, S. F.; Dean, D. W.; Wagman, A. S. *Tetrahedron Lett*. **1992**, *33*, 1839.
17. All assays were carried out with a mixure (1:1) of the diastereoisomers.
18. Nara, F.; Tanaka, M.; Hosoya, T.; Suzuki-Konagai, K.; Ogita, T. *J. Antibiot*. **1999**, *52*, 525.
19. Nara, F.; Tanaka, M.; Masuda-Inoue, S.; Yamasato, Y.; Doi-Yoshioka, H.; Suzuki-Konagai, K.; Ogita, T. *J. Antibiot*. **1999**, *52*, 531.
20. The assay methods of SMases and ceramide synthase were as described previously: Soeda, S.; Kominato, T.; Shimeno, H. *Res. Commun. Biochem. Cell Mol. Biol*. **2000**, *4*, 11.
21. Liu, B.; Hannun, Y. A. *J. Biol. Chem*. **1997**, *272*, 16281.
22. Schissel, S. L.; Keesler, G. A.; Schuchman, E. H.; Williams, K. J.; Tabas, I. *J. Biol. Chem*. **1998**, *273*, 18250.
23. PC-12 cells were obtained from RIKEN cell bank and neuronally differentiated with nerve growth factor.
24. Hannun, Y. A. *J. Biol. Chem*. **1994**, *269*, 3125.
25. Crowe, M. J.; Bresnahan, I. C.; Shuman, S. L.; Masters, J. N.; Beattie, M. S. *Nat. Med*. **1997**, *3*, 73.
26. Hill, I. E.; MacManus, J. P.; Rasquinha, I.; Tuor, U. I. *Brain Res*. **1995**, *676*, 398.
27. Pettmann, B.; Henderson, C. E. *Neuron* **1998**, *20*, 633.