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Bioorganic & Medicinal Chemistry Letters
Bioorganic & Medicinal Chemistry Letters 15 (2005) 2141–2144

Synthesis of sphingomyelin sulfur analogue and its behavior toward sphingomyelinase

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Received 8 January 2005; revised 5 February 2005; accepted 5 February 2005
Available online 19 March 2005

Abstract—The sulfur analogue of sphingomyelin was designed and stereoselectively synthesized from *S*-benzyl-*N*-Boc-cysteine. The introduction of the phosphoryl choline moiety was successfully achieved by our own method using 2-bromoethyl dimethyl phosphite and carbon tetrabromide followed by a trimethylamine treatment. The synthesized compound proved to be a useful substrate for monitoring the enzyme activity of sphingomyelinase by detecting the liberated thiol group with a thiol-sensitive reagent.

1. Introduction

Previously, we reported the syntheses of the carbon and nitrogen analogues of sphingomyelin, in which the phosphonate oxygen to be hydrolyzed by sphingomyelinase (SMase) was replaced by carbon[1] and nitrogen atoms,[2] respectively. These new analogues showed moderate inhibitory activities toward *Bacillus cereus* SMase. Generally, SMase produces phosphoryl choline and ceramide resulting from the specifically catalyzed hydrolysis of the phosphoester linkage of sphingomyelin.[3] The produced ceramide has been known as a lipid second messenger in mammalian cell membranes and plays key roles in the cellular signal transmission pathway, in particular, as a signal transduction factor in cell differentiation and apoptosis derivation (Fig. 1).[4] To elucidate the detailed catalytic mechanism of SMase, development of sphingomyelin analogues that competitively act at the catalytic site and strongly inhibit the hydrolytic ability of the enzyme, has strongly been desired.[5]

In this paper, we describe the highly efficient and stereocontrolled synthesis of the short chain sulfur analogue **1** (Fig. 2) and its behavior toward *B. cereus* SMase.

A sulfur analogue would behave in a manner similar to that for the original sphingomyelin, since sulfur and oxygen atoms possess very similar orbital arrangements particularly in the s and p orbitals. Therefore, the analogue **1** was expected to act as an inhibitor or as a substrate for SMase.

For example, a sulfur analogue of glycerophospholipids was reported by Martin's group[6] to be a strong inhibitor toward phosphatidylcholine-specific phospholipase C (PC-PLC). On the other hand, phosphatidylinositol-specific phospholipase C (PI-PLC)[7] and phospholipase A_2 (PLA_2)[8] were shown to be capable of cleaving the S–P bond of the thiophosphate linkage in addition to the O–P bond of the phosphoester linkage. Therefore, sulfur analogues have been used as substrates for continuous spectrophotometric assays of PI-PLC and PLA_2, in which the release of the free thiol could be followed by the coupling reaction with a colorimetric thiol reagent.[9] A number of methods to measure SMase activity have been developed, and NBD-labeled sphingomyelin,[10] 2-hexadecanoylamino-4-nitrophenylphosphorylcholine,[11] and ^{14}C-labeled sphingomyelin[12] have often been used as substrates. Unlike these compounds, the analogue **1** might have the possibility to be hydrolyzed by SMase, and might be useful as a substrate for the continuous spectrophotometric assay.

Keywords: Sphingomyelinase; Substrate analogue.
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doi:10.1016/j.bmcl.2005.02.020

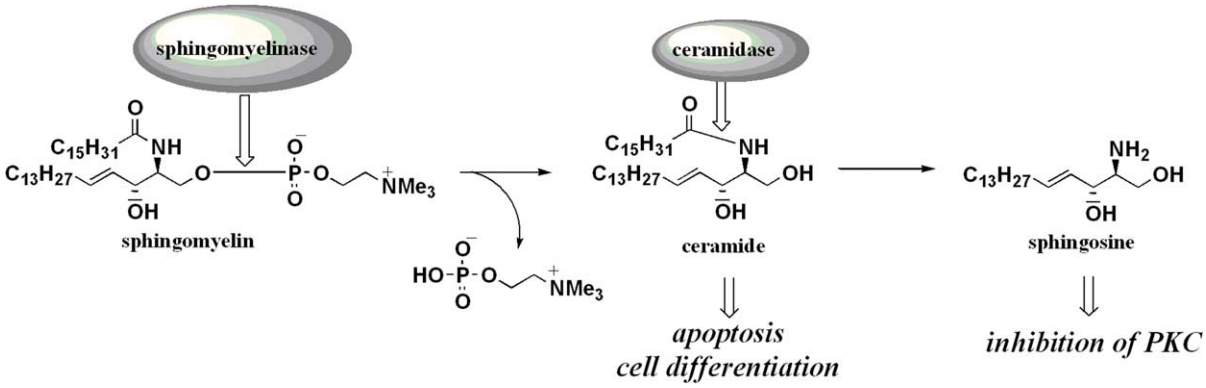


Figure 1. Sphingolipid catabolite.

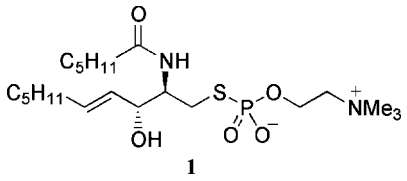
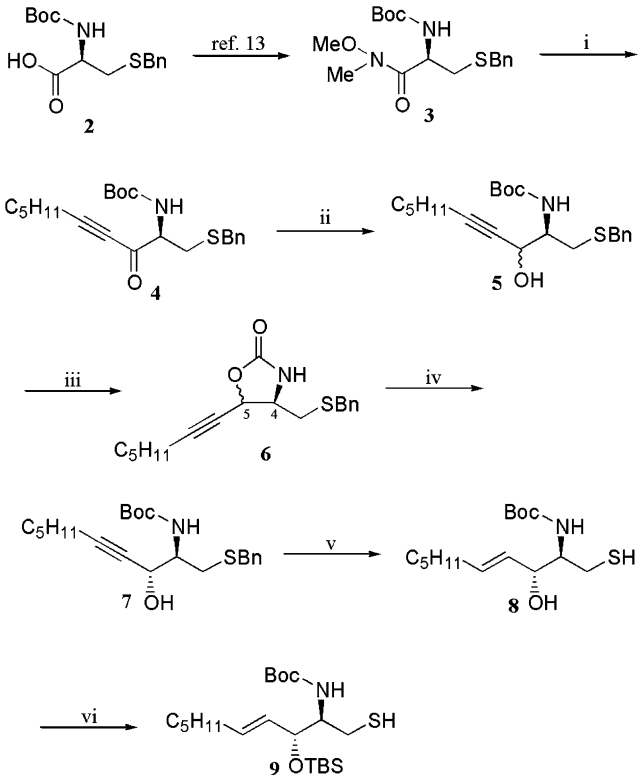


Figure 2. Designed sulfur analogue of sphingomyelin.

2. Results and Discussion

In order to achieve the efficient synthesis of **1**, we started the synthesis from the Weinreb amide **3**, which was easily prepared from *S*-benzyl-*N*-Boc-cysteine **2** according to the literature[13] in 80% yield (Scheme 1).

The monoalkynylation of the amide successfully proceeded at −43 °C with 3 equiv of lithium acetylide to afford **4** in 80% yield. The *anti*-selective reduction of this ketone **4** was investigated. Reduction with diisobutylaluminum 2,6-di-*tert*-butyl-4-methylphenoxide,[14] diisobutylaluminum hydride, and Red-Al gave a 4:1, 3:1, and 5:2 mixture of the *anti*- and *syn*-derivatives, respectively. Reduction with sodium borohydride produced no selectivity. When lithium tri-*tert*-butyloxyaluminohydride, however, was employed in ethanol at −78 °C,[15] the selectivity was improved to 6:1 by NMR, and both isomers were not separable at this stage. The reduction products were transformed into the corresponding oxazolidinone derivative **6** by a sodium hydride treatment in THF at 50 °C. The stereochemistry of the major product was confirmed to be a *syn*-orientation concerning the two substituents on the oxazolidinone ring by comparing the J_{4-5} value of the oxazolidinone derivative **6**; J_{4-5} of the major product was 7.8 Hz, and that of minor product was 5.8 Hz.[16] The undesired stereoisomer could be separated by simple column chromatography after introduction of a *tert*-butoxycarbonyl group into the carbamate nitrogen of **6**. The pure oxazolidinone derivative thus obtained was hydrolyzed by treatment with cesium carbonate in methanol to give **7**.[17] The treatment of **7** with lithium in liquid NH_3 under reflux produced the desired thiol **8** in 93% yield resulting from the reduction of the triple bond and removal of the protecting group at the primary mercapto group. In this reaction, the corresponding dimeric disulfide was often produced

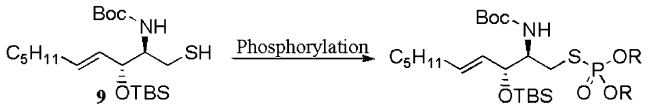


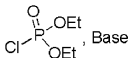
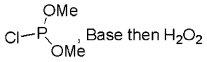
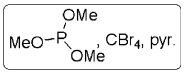
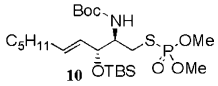
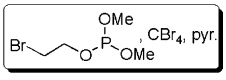
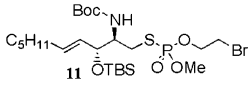
Scheme 1. (i) $C_5H_{11}CCLi$, THF, −43 °C; (ii) $LiAl(OBu)_3H$, EtOH, −78 °C; (iii) NaH, THF, 50 °C; (iv) (a) Boc_2O, DMAP, Et_3N, DMF, 0 °C; (b) separation; (c) Cs_2CO_3, MeOH, 47% for six steps; (v) Li, liq. NH_3, THF, reflux, 93%; (vi) (a) TBSCl, DMAP, Et_3N, DMF, 73%; (b) TBAF, THF, −78 °C, quant.

and the yield of the desired compound was lowered, unless a careful workup under nitrogen was employed. In order to introduce a phosphoryl choline group into the primary mercapto group, protection of the secondary hydroxy group was necessary. Fortunately, after protection of both the mercapto and hydroxy groups of **8** as *tert*-butyldimethylsilyl ethers, selective deprotection of the primary mercapto group was successful by treatment with TBAF in THF at −78 °C to quantitatively produce **9**.

With the desired thiol **9** in hand, the introduction of a phosphorylcholine group into the mercapto group was examined. In our previous synthesis of sphingomyelin,

Table 1. Phosphorylation

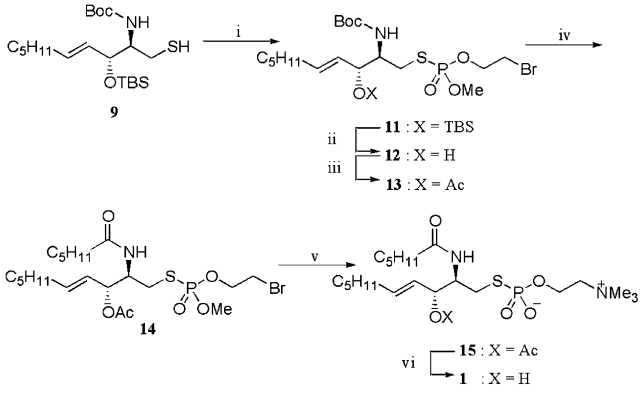


Conditions	Yield	Products
	No reaction or <5%	
		Unidentified product
	57%[a]	
	Quant.	

[a] The reaction conditions were not optimized.

this group was successfully introduced with cyclic chlorophosphate in the presence of triethylamine.[18] Under this condition, however, the phosphorylation of **9** proceeded only with a low yield. After various attempts as shown in Table 1,[19] the phosphorylation of **9** was realized by treatment with trimethyl phosphite **A** and carbon tetrabromide[20] to afford the corresponding dimethylester **10** in 57% yield. Based on the obtained results, we designed 2-bromoethyl dimethyl phosphite **B** instead of trimethyl phosphite **A** and expected to obtain the desired compound **11**, which would be easily transformed into the desired choline moiety. Compound **B** was actually prepared by the reaction of methyl dichlorophosphite with 2-bromoethanol and methanol in the presence of diisopropylethylamine in THF at −78 °C,[21] and compound **2** was treated with a mixture of **B** and carbon tetrabromide in pyridine at 0 °C to successfully and quantitatively produce the corresponding **11**. Moreover, the obtained compound **11** showed an ordinary polarity, and its purification and transformation were much easier than the compounds containing the highly polar phosphorylcholine moiety.

The remaining issues from **11** to **1** were the introduction of an acyl group at the nitrogen group and formation of the choline moiety (Scheme 2). Transformation of the secondary hydroxy protecting group into an acetyl group, subsequent removal of the *tert*-butoxycarbonyl group, and introduction of an acyl group onto the resulting primary amine afforded **14**, which was treated with anhydrous trimethylamine in toluene in a pressure bottle at 60 °C to quantitatively give the desired choline compound **15**. Finally, deprotection of the secondary hydroxy group produced the desired compound **1** after purification by reverse phase HPLC. Thus, the stereoselective synthesis of the sulfur analogue **1** was efficiently achieved from cysteine.[22]



Scheme 2. (i) **B**, CBr_4, pyr, 0 °C, quant.; (ii) 2N HCl, MeOH; (iii) Ac_2O, pyr; (iv) TFA, CH_2Cl_2, then 1N NaOH, $C_5H_{11}COCl$, 88%; (v) Me_3N, toluene, 95%; (vi) KOH, MeOH, 90%.

The inhibitory activity of analogue **1** toward *B. cereus* SMase was tested,[23] and we found that this compound seemed to inhibit the enzyme activity much weaker than the previous carbon and nitrogen analogues of sphingomyelin,[1,2] but was hydrolyzed by this enzyme to release a free thiol. This compound **1** appeared to be a useful substrate for monitoring the enzyme activity of SMase, because the liberated thiol group easily reacts with a thiol-sensitive reagent such as 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB) to form the yellow dianion of 5-thio-2-nitrobenzoic acid (TNB), which can be continuously measured spectrophotometrically.[9] Figure 3 shows the time-course of the increases in absorbance after the addition of *B. cereus* SMase in the presence of several concentrations of the mixed micelle of the sulfur analogue **1** with Triton X-100 (the molar ratio of 1:10). The result showed that the sulfur analogue **1** was hydrolyzed by SMase and the initial velocities could be determined. Thus, the sulfur analogue **1** proved to be a useful substrate for the SMase assay.

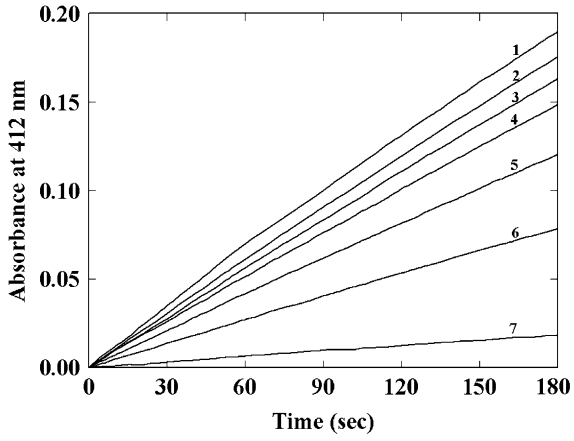


Figure 3. Enzymatic hydrolysis of mixed micelle of sulfur analogue 1 with Triton X-100 (the molar concentration of 1:10) by *B. cereus* SMase. The analogue concentrations: (1) 1.6; (2) 1.2; (3) 1.0; (4) 0.8; (5) 0.6; (6) 0.4; (7) 0.2 mM.

3. Assay methods

An assay solution containing the appropriate concentrations of the mixed micelle of the sulfur analogue **1** with Triton X-100 (the molar ratio of 1:10) at the ionic strength of 0.2 and pH of 7.5 was placed in a semimicro UV cuvette thermostatted at 37 °C. After equilibration of the assay solution for 5 min, a solution of *B. cereus* SMase (5 μL) was added, and the absorbance at 412 nm was recorded for 3 min. The final concentrations of $MgCl_2$, *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid (TES), and DTNB in the assay solution were 10, 50, and 0.83 mM, respectively.

Acknowledgements

This work was partially supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science, Sports and Culture of Japan. T.H. is grateful to the JSPS for a Research Fellowship for Young Scientists.

References and notes

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21. The detailed results will be reported elsewhere.
22. Data for **1**: $[\alpha]_D^{23.0} - 9.2$ (*c* 0.55, CH_3 OH); IR (NaCl neat) 3418, 2928, 1640, 1547, 1229, 1073 cm^{-1}; ^1H NMR (CD_3OD, 400 MHz) δ 5.70 (td, $J = 6.8$, 15.1 Hz, 1H), 5.45 (mdd, $J = 7.1$, 15.4 Hz, 1H), 4.28–4.30 (m, 2H), 4.02 (dd, $J = 7.1$, 7.8 Hz, 1H), 3.97 (ddd, $J = 3.7$, 6.8, 8.8 Hz, 1H), 3.62–3.72 (m, 2H), 3.24 (s, 9H), 3.13 (ddd, $J = 3.7$, 12.2, 13.7 Hz, 1H), 2.83 (ddd, $J = 8.8$, 13.7, 14.9 Hz, 1H), 2.19 (t, $J = 6.8$ Hz, 2H), 2.03 (td, $J = 7.1$, 7.1 Hz, 2H), 1.61 (tt, $J = 7.3$, 7.3 Hz, 1H), 1.28–1.42 (m, 10H), 0.92 (t, $J = 6.8$ Hz, 3H), 0.90 (t, $J = 6.8$ Hz, 3H); ^{13}C NMR ($CDCl_3$, 100 MHz): δ 176.2, 134.9, 130.8, 75.1, 67.5 (m), 60.5 ($J_{C-P} = 5.0$ Hz), 56.1 ($J_{C-P} = 2.5$ Hz), 54.78, 54.75, 54.7, 49.0, 49.4, 49.2, 49.0, 48.8, 48.6, 48.4, 37.3, 33.4, 32.6 (3C), 30.0, 26.9, 23.6, 23.5, 14.4, 14.3; FAB HRMS *m/z* calcd for $C_{21}H_{44}N_2O_5PS$ (M^++H) 467.2708, found 467.2718.
23. Sequence comparison of *B. cereus* SMase with mammalian neutral Mg^{2+}-dependent SMases (*n*SMases) revealed that the amino acid residues important for Mg^{2+} binding have been conserved. We are planning the hydrolysis experiment of sulfur analogue by *n*SMases.