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New Ganglioside Analogs that Inhibit Influenza Virus Sialidase

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Synthetic thioglycoside-analogs of gangliosides such as Neu5Acα(2-*S*-6)Glcβ(1-1)Ceramide (1) and the GM3 analog Neu5Acα(2-*S*-6)Galβ(1-4)Glcβ(1-1)Ceramide (2), competitively inhibited GM3 hydrolysis by the sialidase of different subtypes of human and animal influenza viruses with an apparent K_i value of 2.8×10^{-6} and 1.5×10^{-5} M, respectively. The inhibitory activity of the ganglioside GM4 analog [Neu5Acα(2-*S*-6)Galβ(1-1)Ceramide (3)], in which the glucose of 1 was substituted by galactose, was lower than that of 1 ($K_i = 1.0 \times 10^{-4}$ M). The thioglycoside-analogs (1, 2, 3) of the gangliosides were non-hydrolyzable substrates for influenza virus sialidase. The inhibitory activity of 1 to bacterial sialidases from *Clostridium perfringens* and *Arthrobacter ureafaciens* was considerably lower than that to influenza virus sialidase, indicating that the structure of the active site in bacterial and influenza virus sialidase may be different and the analogs may be useful to determine the orientation of the substrate to the active site of sialidases, especially of influenza viruses.

Cell membrane gangliosides, sialic acid containing glycosphingolipids, have biological roles such as cell growth, differentiation, adhesion, oncogenesis and as cellular receptors for viruses and bacteria (for reviews, see [3-5]). We have reported that some gangliosides exogenously added to sialidase-treated erythrocytes induce cellular responses to influenza A and B viruses [6-9], indicating that gangliosides are cellular receptor sialoglycoconjugates for influenza viruses. The influenza virus sialidase (EC 3.2.1.18; *N*-acetylneuraminyl glycohydrolase) is a spike glycoprotein integrated in viral membranes, identified as the receptor destroying enzyme [10]. This molecule has been crystallized and its three dimensional structure was studied by X-ray crystallography [11, 12]. The sialidase is suggested to play important roles in viral infection and release of virion by budding processes [13]. The sialidase is clustered on the viral membranes, while the hemagglutinin, another

Abbreviations: Cer, ceramide; GM3, Neu5Acα(2-3)Galβ(1-4)Glcβ(1-1)Cer; GM4, Neu5Acα(2-3)Galβ(1-1)Cer. Gangliosides were abbreviated according to Svennerholm [1] and the recommendation of the IUPAC-IUB Commission on Biochemical Nomenclature [2].

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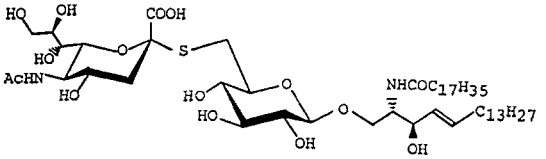
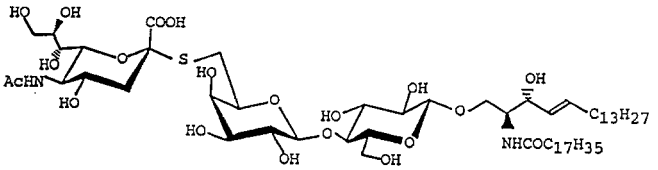


Compound 1



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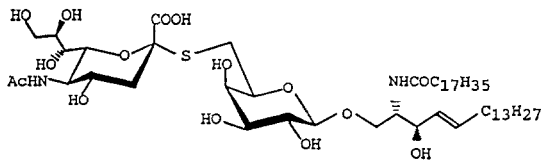

Compound 3

Figure 1. Structure of synthetic thioglycoside-analogs of gangliosides.

spike glycoprotein, is distributed uniformly over the virus surface [14]. The clustered arrangement of sialidase may be advantageous to cleave sialic acid residues from hemagglutinin-receptor complex on the cell membrane near the point of budding, thereby facilitating virus release. Despite all this information, the function of the sialidase is little known. If a potent, influenza virus-specific and stable competitive inhibitor for the sialidase is available, inhibition of the virus infection and also modulation of cellular biological responses may become possible by regulating the sialidase activity.

This report demonstrates new metabolically stable ganglioside analogs containing a thioglycoside linkage which potently and widely inhibits sialidases of different subtypes of influenza viruses.

Materials and Methods

Gangliosides and Thioglycoside Analogs of Gangliosides

Ganglioside GM3 from human liver was isolated as described by Seyfried *et al.* [15]. A series of synthetic ganglioside analogs containing a thioglycosidic linkage; i.e. compound **1** [Neu5Acα(2-*S*-6)Glcβ(1-1)Cer, *S*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2-6)-*O*-(6-thio-β-D-glucopyranosyl)-(1-1)-(2*S*, 3*R*, 4*E*)-2-octadecanamido-4-*O*-octadecene-1,3-diol], compound **2**, a ganglioside GM3 analog [Neu5Acα(2-*S*-6)Galβ(1-4)Glcβ(1-1)Cer, *S*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2-6)-*O*-(6-thio-β-D-galactopyranosyl)-(1-4)-*O*-(β-D-glucopyranosyl)(1-1)-(2*S*, 3*R*, 4*E*)-2-octadecanamido-4-octadecene-1,3-diol], and compound **3**, a ganglioside GM4 analog [Neu5Acα2-*S*-6Galβ(1-1)Cer, *S*-(5-Acetamido-3,5-dideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic acid)-(2-6)-*O*-(6-thio-β-D-glucopyranosyl)-(1-1)-(2*S*, 3*R*, 4*E*)-2-octadecanamido-4-octadecene-1,3-diol], were prepared as described previously [16, 17].

Viruses and Sialidases

Influenza A virus/PR/8/34 (H1N1), A/Japan/305/57 (H2N2), A/Aichi/2/68 (H3N2), A/Equine/Miami/1/63 (H3N8), A/Swine/Iowa/15/30 (H1N1), A/Duck/Ukrine (H3N8) and Newcastle disease virus (D-26 and Miyadera strain) were propagated in 11-day-old embryonic chicken eggs, purified and concentrated as described previously [18, 19]. The virus suspensions in saline were used as sources of sialidase showing enzyme activity with naturally occurring GM3 (one of the most commonly detected gangliosides) as substrate of 2.0 units/mg protein (10 units/ml). *Clostridium perfringens* sialidase was obtained from Sigma Chemical Co., St. Louis, MO, USA (Type V). *Arthrobacter ureafaciens* sialidase was a gift from Dr. Uchida (Marukin Shoyu, Kyoto, Japan). Bacterial sialidases were dissolved in 2 mM sodium acetate buffer (pH.5.2) and used as enzyme preparations.

Enzyme Assays

Sialidase activity was determined as follows: Standard reaction mixtures (total 40 µl) contained 7.5 nmol GM3-ganglioside (final concentration, 1.5×10^{-4} M) as substrate and appropriate amount of sialidase inhibitor (usually 7.5×10^{-5} M) mixed by vortexing in 2 mM sodium acetate buffer (pH 5.2) and pre-incubated for 5 min at 37°C. Enzyme preparation (viral or bacterial sialidase, 10 µl) in the same buffer was added and incubated at 37°C for 10 min. Incubations to determine the reactivity of the thioglycoside analogs of gangliosides to the viral sialidase were performed in the presence or absence of sodium taurodeoxycholate (Sigma; final concentration 0.1%), which was the most effective detergent tested for the activation of influenza virus sialidase activity (data will be published elsewhere). Reactions were terminated by addition of 40 µl methanol and aliquots of the reaction mixture were spotted on a silica gel thin layer plate (Merck, Darmstadt, W. Germany). The plate was developed in chloroform/methanol/water, 60/40/10 by vol. Free and lipid-bound sialic acid was visualized by resorcinol-HCl reagent [20] spray. Free sialic acid migrated near the origin was quantified densitometrically by reading the optical density at 580 nm with a dual wavelength TLC scanner (CS 910, Shimadzu, Kyoto, Japan).

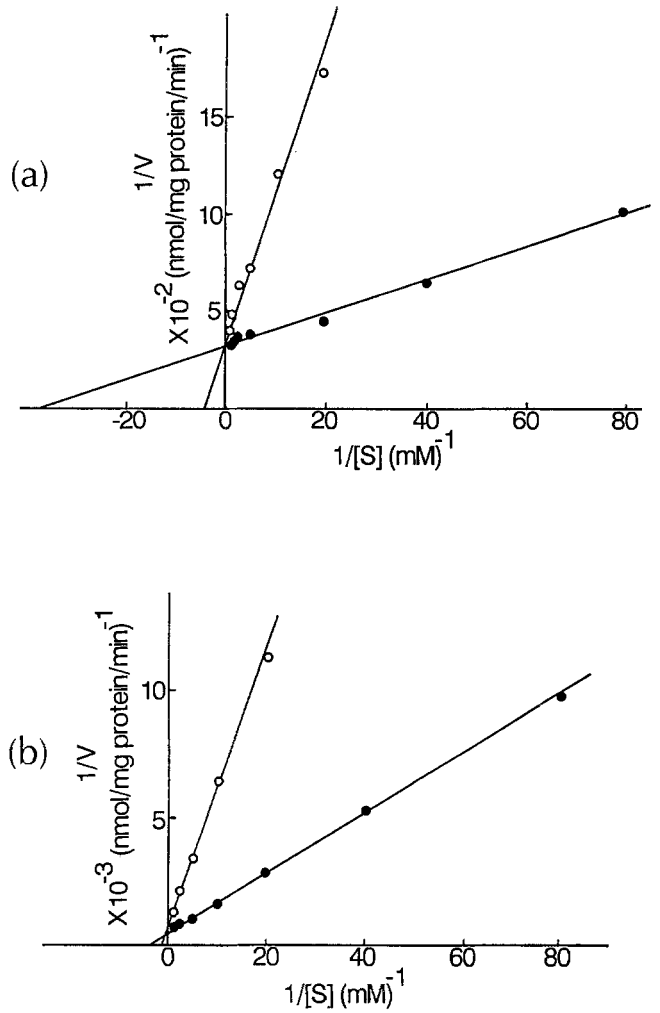

Figure 2. Hydrolysis of GM3-ganglioside by the sialidase of influenza virus [A/PR/8/34 (H1N1)] in the absence (●) and in the presence (○) of 150 x 10^{-6} M of compound **1** (a) or compound **2** (b).

Resorcinol-HCl reagent reacted linearly with free Neu5Ac on silica gel thin-layer plates depending on the amount (0.1-8.0 nmol) of Neu5Ac. Enzyme activity was determined as Neu5Ac released (nmol/mg protein/min). The determination of K_M and V values for GM3-ganglioside was carried out in incubations at different substrate concentrations (12.5 - 1600 μM) with sialidase diluted to give 20-50% release of Neu5Ac within the duration of the incubation. These conditions were determined in preliminary experiments to ensure linearity of release for each enzyme concentration.

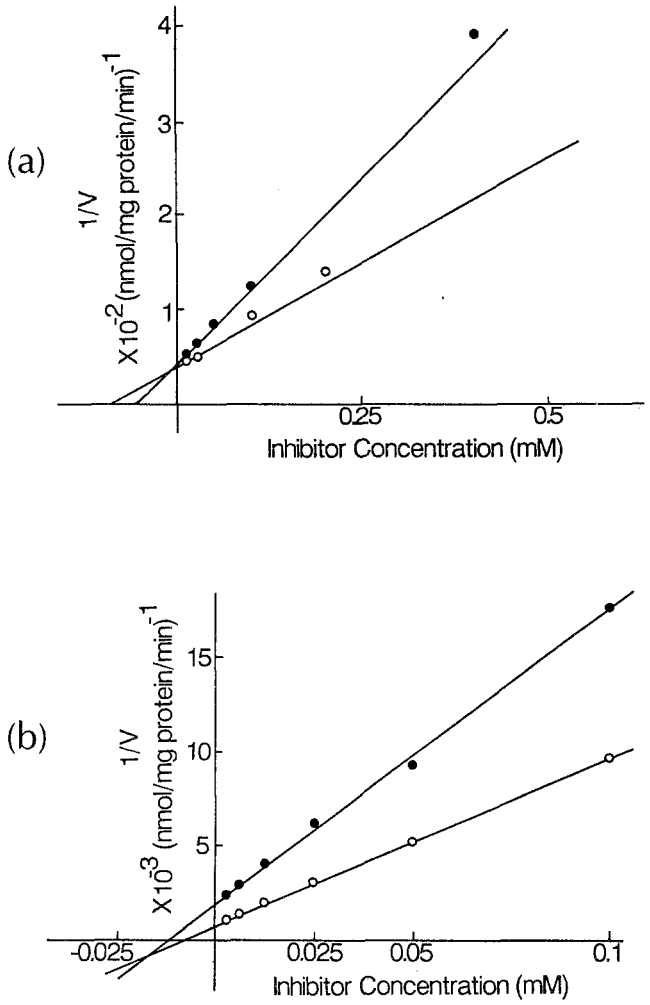

Figure 3. Dixon plot from the results determined by using two different concentration of GM3 substrate (●, 0.1 mM; ○, 0.2 mM) in the presence of increasing amounts of the thioglycoside analog of gangliosides, compound **1** (a) and **2** (b).

Results and Discussion

Inhibitory activity of synthetic thioglycoside-analogs of gangliosides, compound **1** [NeuAcα(2-*S*-6)Glcβ(1-1)Cer] and compound **2**, a ganglioside GM3 analog [Neu5Acα(2-*S*-6)Galβ(1-4)Glcβ(1-1)Cer] (see Fig. 1) for the influenza virus A/PR/8/34 (H1N1) sialidase was examined. Lineweaver-Burk plots obtained from the data on the hydrolysis of GM3-gangliosides in the presence or absence of the inhibitors **1** and **2** apparently showed that **1**

Table 1. Reactivity of thioglycoside analogs of gangliosides by the sialidase of influenza virus [A/PR/8/34 (H1N1)]. Reaction mixtures (total 40 µl) containing 7.5 nmol of each substrate (GM3-ganglioside, compound **1**, **2** or **3**; final concentration, 1.5×10^{-4} M) mixed with 2 mM sodium acetate buffer (pH 5.2) were pre-incubated at 37°C for 5 min in the presence or absence of sodium taurodeoxycholate (final concentration 0.1%), followed by addition 10 µl of enzyme preparation suspended in the same buffer, and incubation at 37°C for 10 min. Enzyme activity was determined as described in the Materials and Methods section.

Substrates	Enzyme activity (nmol/mg protein/min)	
	without TDC[a]	with TDC
GM3	473 (34.2)[b]	1380 (100)
Compound **1**	0	25 (1.8)
Compound **2**	0	26 (1.9)
Compound **3**	0	22 (1.6)

[a] TDC = Sodium taurodeoxycholate.
[b] % Hydrolysis is given in parentheses.

and **2** were competitive inhibitors of the influenza virus sialidase (Fig. 2). The apparent K_M values of A/PR/8/34 (H1N1) sialidase found in this study with GM3-ganglioside show 1.6×10^{-5} M in the absence of the inhibitor **1** while an approximately 16-fold increase of K_M value (2.6×10^{-4} M) was observed in the presence of 200 mM of **1** without changing the V_{max} value. The property as competitive inhibitor was also confirmed by a Dixon plot calculated from the results determined by using two different concentrations of GM3 substrate (0.1 mM, 0.2 mM) in the presence of increasing amounts of the inhibitor (Fig. 3). The apparent K_i values of **1** and **2** were determined as 2.8×10^{-6} M and 1.5×10^{-5} M, respectively, indicating that **1** is a more potent inhibitor than **2**. It is worth noting that the inhibitory activity of compound **3** ($K_i = 1.0 \times 10^{-4}$ M), a ganglioside GM4 analog in which the β-D-glucose of **1** was replaced by β-D-galactose [NeuAcα(2-*S*-6)Galβ(1-1)Cer, see Fig. 1] and **2**, the GM3 analog carrying β-D-galactose at the penultimate position, were lower than that of **1**. The above results indicate that the conformation of the hydroxyl group at C-4 of the penultimate sugar and/or oligosaccharide chain length may be important for the substrate recognition by influenza virus sialidase.

Compounds **1**, **2** and **3** were all non-hydrolyzable substrates for influenza virus sialidase under the standard reaction conditions without detergent, and were stable even under the reaction conditions which allowed complete hydrolysis of GM3-ganglioside in the presence of sodium taurodeoxycholate, the most effective detergent tested for the hydrolysis of GM3-ganglioside by influenza virus sialidase (Table 1).

Table 2. Inhibition of the sialidases of various subtypes of influenza virus and Newcastle disease virus. The reaction mixtures (50 µl) containing GM3-ganglioside (1.5×10^{-4} M) as substrate were incubated in the presence or absence of compound **1** (7.5×10^{-5} M) as described in the text. Inhibitory activity (%) of compound **1** was calculated using the following equation: [(nmol sialic acid hydrolyzed in the absence of inhibitor - nmol sialic acid hydrolyzed in the presence of inhibitor)/ nmol sialic acid hydrolyzed in the absence of inhibitor] x 100.

	Inhibitory activity (%)
Influenza viruses	
A/PR/8/34 (H1N1)	84.0
A/Japan/305/57 (H2N2)	67.1
A/Aichi/2/68 (H3N2)	82.5
A/eq/Miami/1/63 (H3N8)	65.0
A/sw/Iowa/15/30 (H1N1)	59.0
Newcastle disease viruses	
D-26	56.4
Miyadera	63.8

The inhibitory activity for bacterial sialidases from *Clostridium perfringens* and *Arthrobacter ureafaciens* was examined using **1**, the most potent inhibitor for influenza virus sialidase tested. The K_i values for bacterial sialidase (4.9×10^{-5} M for *Cl. perfringens*; 4.0×10^{-4} M for *Ar. ureafaciens*) were 18- and 143-fold higher than that for influenza virus [A/PR/8/34 (H3N2)] sialidase ($K_i = 2.8 \times 10^{-6}$ M), respectively, indicating that **1** preferentially inhibits the viral sialidase compared to bacterial sialidases. The above results also indicate that the structure of the active site for binding sialic acid residues in the substrate GM3 in bacterial and influenza virus sialidases may be different.

Compound **1** widely and potently inhibited the sialidase activities of different sialidase subtypes of human and animal influenza A viruses [A/PR/8/34 (H1N1), A/Japan/305/57 (H2N2), A/Aichi/2/68 (H3N2), A/equine/Miami/1/63 (H3N8), and A/swine/Iowa/15/30 (H1N1)], and also some strains of Newcastle disease viruses (D-26, Miyadera), a group of paramyxovirus containing hemagglutinin-sialidase glycoprotein, at final concentrations in the order of 10^{-5} M (Table 2).

Compound **1** may be useful for crystallographic studies on the binding of sialic acid residue to the active site of influenza virus and related paramyxovirus sialidases, because of the resistant properties from the hydrolysis by sialidase, and also as a new anti-influenza and Newcastle disease virus agent to inhibit viral release from the infected cells. Several kinds of natural and synthetic sialidase inhibitors have been reported. Natural product inhibitors such as DNA, RNA, heparin, dextran sulfate [21], neuraminin (a glycoprotein) [22] and Neu5Ac2en [23] have been reported, while synthetic sialidase competitive inhibitors, analogs of neuraminic acid, such as 2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid and its

derivatives [24, 25], 2-deoxy-2,3-dehydro-*N*-trifluoroacetylneuraminic acid (FANA) [26] have been reported. Thioglycoside analogs of gangliosides described in this paper are a new type of potent inhibitor of sialidases of several kinds of influenza virus.

Acknowledgements

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