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Design, synthesis, and biological evaluation of ganglioside Hp-s1 analogues varying at glucosyl moiety

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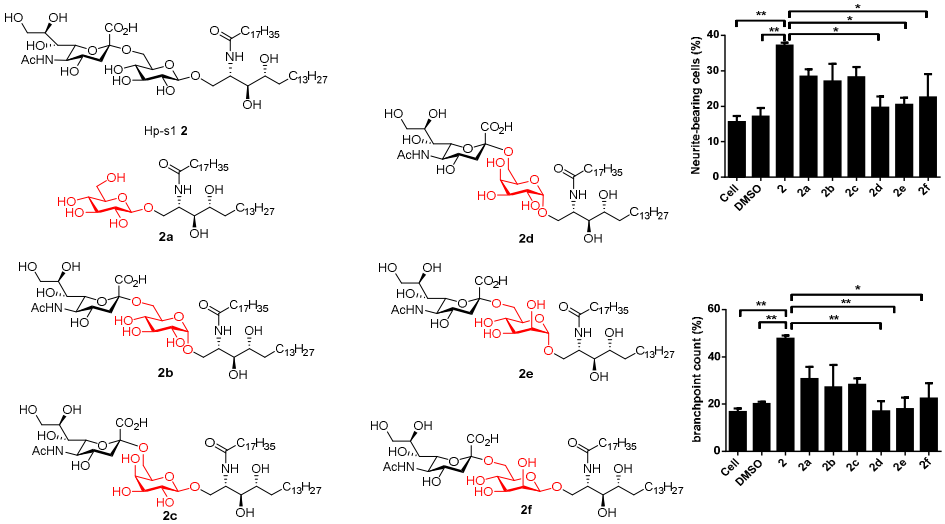
Supporting Information Placeholder



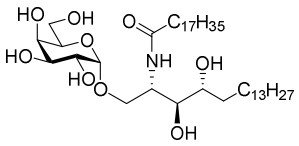
ABSTRACT: Ganglioside Hp-s1 is isolated from ovary of sea urchin *Diadema setosum*. It exhibited better neuritogenic activity than GM1 in pheochromocytoma 12 cells. To explore the roles of glucosyl moiety of Hp-s1 in contributing to the neurogenic activity, we developed feasible procedures for synthesis of Hp-s1 analogues (**2a-2f**). The glucosyl moiety of Hp-s1 was replaced with α-glucose, α-galactose, β-galactose, α-mannose, β-mannose, and their biological activities on SH-SY5Y cells and natural killer T (NKT) cells were evaluated. We found that the orientation of C-2 hydroxyl group at glucosyl moiety of Hp-s1 plays an important role to induce neurite outgrowth of SH-SY5Y cells. Surprisingly, compound **2d** could activate NKT cells to produce interleukin 2, although it did not show great activity on neurite outgrowth of SH-SY5Y cells. In general, the Hp-s1 might be considered as a lead compound for the development of novel drugs aimed at modulating the activity of neuronal cells.

Keywords: Ganglioside Hp-s1, neurogenic activity, SH-SY5Y cells, glucosyl moiety of Hp-s1

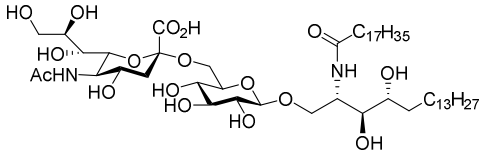
Introduction: Glycosphingolipids play an important role in cell recognition and signaling.[1] Gangliosides, sialic acid-containing glycosphingolipids, are abundant in the central and peripheral nervous systems and are critical to stabilize the various brain functions.[2,3] On the other hand, exogenously administered gangliosides showed neurotrophic and neuritogenic effects on neuronal cells *in vitro* and in experimental animal models.[4] For example, ganglioside GQ1b might trigger the activation of protein kinase on cell surface to increase proliferation[5] and neurite outgrowth of human neuroblastoma cell lines, GOTO and NB-1.[6] GM3 induced neuritogenesis of mouse neuroblastoma Neuro2a cells by decreasing Csk/c-Src ratio in glycosphingolipid-enriched domain and then leading to activation of the c-Src.[7] Treatment of GM1 ganglioside improved the survival of lesioned nigral dopamine neurons in rats.[8] Furthermore, it also ameliorated the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced parkinsonian-like symptoms in nonhuman primates[9] and showed neurologic improvement in patients with acute stroke at the first few days.[10] These evidences suggest that treatment of exogenous neuritogenic gangliosides might serve as therapeutic strategy for neurological diseases.

Although many gangliosides have been extracted and identified from echinoderms, such as GP-2, LG-2 and

GAA-7 from starfish, SJG-2, HPG-1 and HLG-3 from sea cucumber, and CJP2, CJP3 and CJP4 from feather star,[11] only a few of them had been reported to have biological activities in neuronal cells. GP-2, which was isolated from starfish *Asterina pectinifera*, showed better activity than GM1 to support the survival of cultured cerebral cortex cells. GAA-7, which is the main oligosaccharide moiety in *Asterias amurensis versicolor,* had neuritogenic and growth-inhibitory activities on mouse neuroblastoma cell line Neuro 2a.[12] Neuritogenic activity of SJG-2, LLG-3 and GAA-7 on rat pheochromocytoma cell PC-12 was enhanced in the presence of nerve growth factor (NGF) when compared with GM1.[2,13] In addition, DSG-A, which was extracted from ovary of sea urchin *Diadema setosum*, displayed higher neuritogenic activity than GM1 in the presence of NGF on PC-12 cells.[14] Because of limited natural resources and difficulties in extraction procedures, chemical synthesis of these neuritogenic gangliosides, such as GAA-7[15] and DSG-A,[16] has drawn attentions of chemists. We were interested in ganglioside Hp-s1 (Figure 1), which was discovered in the sperm of sea urchin *Hemicentrotus pulcherrimus* and ovary of sea urchin *Diadema setosum*.[17] Hp-s1 (Neu5Acα2→6Glcβ1→1Cer) is composed of three major components, including phytosphingolipid, glucosyl moiety, and sialic acid. An Hp-s1 analogue with shorter phytosphingosine chain was synthesized and showed neuritogenic activity on human neuroblastoma cell line SH-SY5Y without NGF,[18] suggesting that the neuritogenic activity of Hp-s1 might have been enhanced through modification of its glycan moiety or ceramide part. Recently, we have shown a straightforward protocol for the synthesis of Hp-s1.[19b] To further explore the role of glucosyl moiety of Hp-s1 on neuritogenic activity, in this study, we synthesized Hp-s1 analogues **2a-2e** with substitution of the glucosyl moiety with other hexopyranoses and evaluated their neuritogenic activity *in vitro*.



α-galactosyl ceramide **1**



ganglioside Hp-s1 **2**

Figure 1. Structures of ganglioside α-galactosyl ceramide **1** and Hp-s1 **2**

Result and Discussion: Recently, we have developed a concise synthesis of α-galactosyl ceramide from D-lyxose in five steps[19a] and a straightforward method for the synthesis of ganglioside Hp-s1 **1** starting from phytosphingosine or D-lyxose in ten steps.[19b] In this study, the ganglioside Hp-s1 **2** was served as a lead molecule and its analogues **2a-2f** (Figure 2) were synthesized as described below. The glucosyl moiety of Hp-s1 was replaced with α-glucopyranoside, α-galactopyranoside, β-galactopyranoside, α-mannopyranoside and β-mannopyranoside. The sialic acid moiety of Hp-s1 was removed to generate **2a**. The compound **2b** was prepared by coupling a glucosyl moiety with phytosphingosine in α-configuration, which is different from Hp-s1 in β-configuration. To evaluate the role of 4-OH position of glucosyl moiety, we synthesized compound **2c** (β-configuration) and **2d** (α-configuration) by using galactosyl moiety. To reveal the character of 2-OH position of glucosyl moiety, compound **2e** and **2f** synthesized via a coupling reaction between mannosyl group and phytosphinogosine at α-configuration and β-configuration respectivly.

By following the literature procedure, the phytosphingosine derived acceptor **3**[19b] and glycosyl donors **4**-**8**[20] were synthesized.

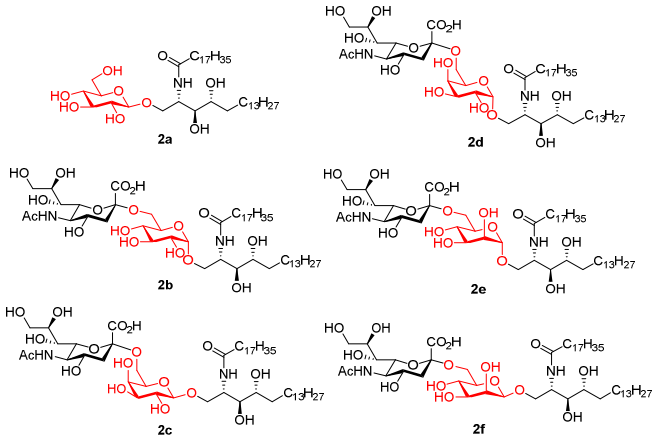


Figure 2. Structures of ganglioside Hp-s1 analogues **2a**–**2f**.

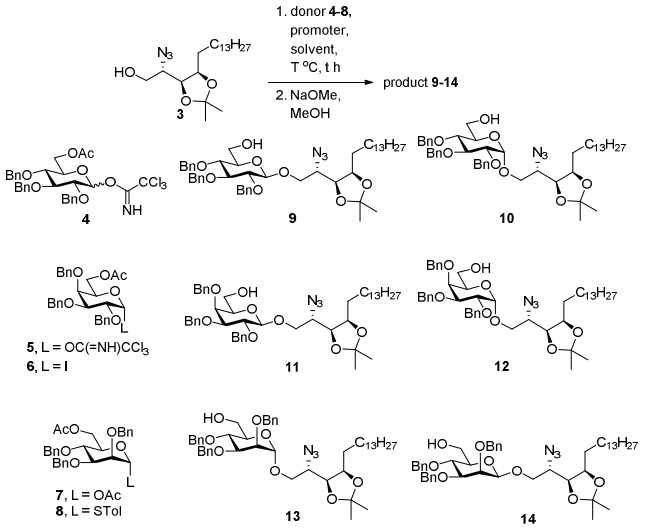
Then, various anomeric leaving groups, such as iodo, acetate, imidate, and STol, were conjugated to glycosyl donors. The compound **3** was glycosylated with donor **4**, promoted by TMSOTf in CH_2Cl_2:CH_3CN solvent at -30 °C to room temperature (25 °C) and followed by hydrolysis to afford β-glucosyl azido compound **9**[19b] in 82% yield (α/β = 1/3.2, Entry 1, table 1). The glycosylation product of acceptor **3** and donor **4** showed similar polarity with starting reagents and α-isomer. Separation of the compound **9** was easily attained through column chromatography by hydrolyzing *O*-6 acetyl group of glucosyl moiety immediately after the first glycosylation. The α-glucosyl azido compound **10** was synthesized by coupling imidate donor **4** with acceptor **3** in CH_2Cl_2 and TMSOTf as promoter, followed by hydrolysis to get 83% yield (α/β = 3.8/1, Entry 2, table 1). β-Galactosyl azido compound **11** was synthesized by treating imidate donor **5** with acceptor **3** in the presence of TMSOTf in mixed solvent CH_2Cl_2:CH_3CN (1:2) and hydrolyzed with NaOMe in MeOH to get 90% yield, with improvement in α/β ratio to 1/5 (Entry 3, Table 1). Best condition for synthesis of α-galactosyl azido compound **12,** (Entries 4-6, Table 1), was rendered when ac-

ceptor **3** was treated with 2.0 equiv. of iodide donor **6** under the influence of TBAI, DIPEA, metallic Cu in toluene at 80 °C and successive removal of *O*-6 acetyl group to afford 85% yield (α/β = 7.5/1, Entry 6, Table 1).

When acetate donor **7** was treated with acceptor **3** in the presence of $BF_3{\cdot}OEt_2$ in CH_2Cl_2 as solvent at 25 °C and hydrolysed with NaOMe to provide α-mannosyl azido compound **13** in 53% yield. Compound **14** was synthesized in 62% by reacting acceptor **3** with donor **8** in CH_2Cl_2 in presence of BSP, DTBMP and Tf_2O reagents followed by hydrolysis (Entries 8-9, Table 1)[20]. All synthons **9-14** were purified with flash chromatography to separate α-isomer and β-isomer. The intermediate **9** was reduced to amine via Staudinger reaction[22] followed by coupling of stearic acid in EDC, HOBt reagents to provide the expected compound **15** in 39% yield. Then, it underwent debenzylation by Pd/C, H_2 and removal of acetonide group by 1N HCl to get β-glucosyl ceramide **2a** in 30% yield (Scheme 1).

Synthesis of intermediates **17-21** is shown in Table 2 by following our previously developed procedures.[19b,23] In brief, glycosylation of acceptor **10** with sialyl donor **16** in CH_2Cl_2:CH_3CN (1/2) at -30°C in the presence of NIS, TfOH, 3Å MS afforded the disaccharide **17** in 65% yield with α/β ratio = 3.6/1 (Entry 1, Table 2). The intermediate compounds **11**, **12**, **13**, and **14** were reacted in a similar fashion with **16** to afford **18** (56% yield, α/β = 1.6/1), **19** (62% yield, α/β = 3/1), **20** (79% yield, α/β = 2.6/1), and **21** (85% yield, α/β = 2.9/1) respectively. The mixtures of α- and β-configuration of compounds **15-18** were separated by column chromatography and only α isomers were taken for the next step (Entries 2-5, Table 2).

Table 1. First glycosylation reactions between acceptor **3** and donors **4-7**.



Entry	Donor	Promoter	Solvent	T (°C)	Product yield, α/β
1	**4**[a]	TMSOTf	CH_2Cl_2/CH_3CN (1/2)	-30 to rt	**9** 82%, 1/3.2
2	**4**[a]	TMSOTf	CH_2Cl_2	rt	**10** 83%, 3.8/1
3	**5**[a]	TMSOTf	CH_2Cl_2/CH_3CN (1/2)	-30 to rt	**11** 90%, 1/5.0
4	**6**[a]	TBAI, DIPEA, Cu	Toluene	65 °C	**12** 45%, 2.0/1
5	**6**[b]	TBAI, DIPEA, Cu	Toluene	65 °C	**12** 68%, 1.8/1
6	**6**[b]	TBAI, DIPEA, Cu	Toluene	80 °C	**12** 85%, 7.5/1
7	**7**[a]	$BF_3 \cdot OEt_2$	CH_2Cl_2	rt	**13** 53%, α only
8	**8**[b]	BSP, DTBMP, Tf_2O	CH_2Cl_2	-60 to rt	**14** 65%, 1/1.0
9	**8**[c]	BSP, DTBMP, Tf_2O	CH_2Cl_2	-60 to rt	**14** 62%, 1/1.5

[a] acceptor/donor = 1 equiv./1.5 equiv.
[b] acceptor/donor = 1 equiv./2.0 equiv.
[c] acceptor/donor = 3.0 equiv./1 equiv.

Azides were reduced by Staudinger reaction to produce free amines and solvent was distilled off under reduced pressure to obtain crude product, and then subsequently dried under high vacuum to remove water. No purification was carried out at this stage and the crudes directly taken for acid-amine coupling with commercially available stearic acid by using EDC, HOBt in anhydrous CH_2Cl_2.

Scheme 1. Preparation of ganglioside Hp-s1 analogues **2a**

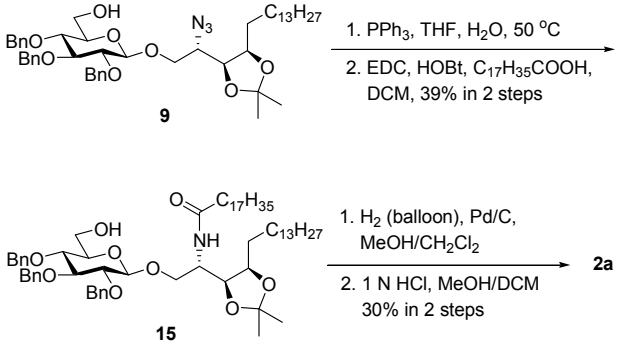
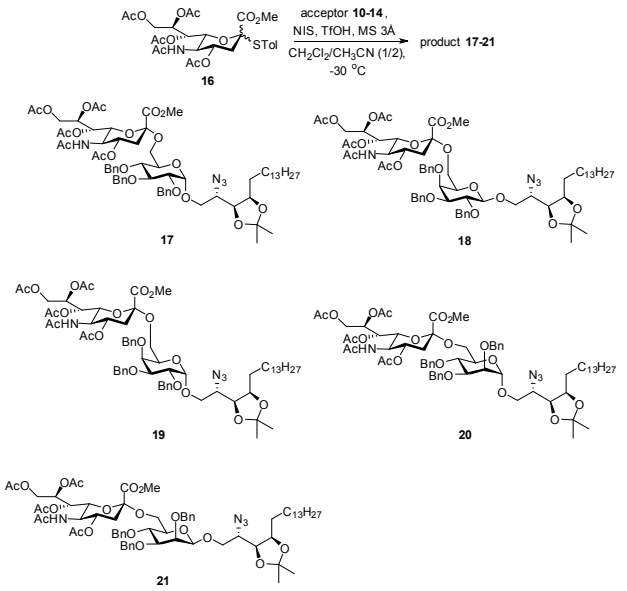


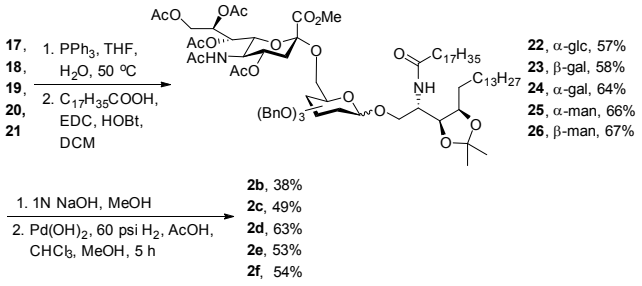
Table 2. Glycosylation of acceptors **10-14** with donor **16**.



Entry	Acceptor	Product	Yield (α/β)
1	**10**	**17**	65% (3.6/1)
2[a]	**11**	**18**	56% (1.6/1)
3	**12**	**19**	62% (3.0/1)
4	**13**	**20**	79% (2.6/1)
5	**14**	**21**	85% (2.9/1)

[a] α/β ratio was confirmed by NMR.

Scheme 2. Finalizing synthesis of ganglioside Hp-s1 analogues **2b**-**2f**.



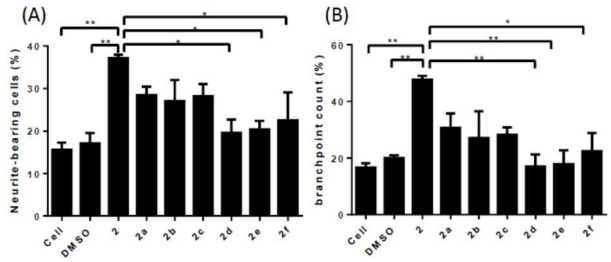


Figure 3. Effect of Hp-s1 analogues on neurite outgrowth of SH-SY5Y cells. Cells were incubated with indicated Hp-s1 analogues (10 μM) for 72 hours, and then percentage of neurite bearing cells (A) and percentage of branch point count (B) were determined. Three independent experiments were conducted and the results were presented as mean of three experiments ± SEM. * P values < 0.05, ** P value < 0.01 and *** p < 0.001 were considered significant.

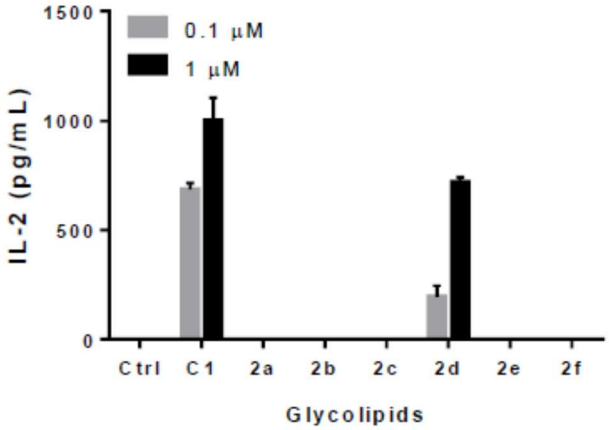


Figure 4. Induction of IL-2 by Hp-s1 analogues in mouse NK1.2 cells. CD1d-expressing A20 cells were loaded with 0.1 or 1 uM of alpha-galactosylceramide (C1) and Hp-s1 analogues, and then cultured with mouse NK1.2 cells for three days. After culture, supernatant was collected to determine the secretion of IL-2 by ELISA. Data were presented as mean ± SD.

As shown in scheme 2, the azide group of **17**-**21** were reduced to amine, and then resulting amine was coupled with acid to get amides **22**-**26** with yields of 57%, 58%, 64%, 66%, and 67% respectively. These compounds **22**-**26** were treated with 1N NaOH in MeOH for deacetylation and debenzylation was achieved by using $Pd(OH)_2$, AcOH, $CHCl_3$, MeOH under 60 psi H_2 pressure to furnish the target molecules **2b, 2c, 2d, 2e,** and **2f** in 38%, 49%, 63%, 53%, and 54% yields respectively.

Biological activity of Hp-s1 analogues

The effects of Hp-s1 **2** and its analogues **2a**-**2f** on neurite outgrowth of human neuroblastoma cell SH-SY5Y were determined. SH-SY5Y cells were treated with Hp-s1 and its analogues for 72 hours. After treatment, the neurite-bearing cells and branch point were counted. As shown in figure 3A, the percentage of neurite-bearing cell was significantly higher in Hp-s1 **2** treated cells (37.1 ± 0.8) than in untreated (15.6 ± 1.6, p=0.0013) and DMSO-treated cells (17.1 ± 2.4, p=0.0028). Compound **2b** (27 ± 4.9, p=0.3) and **2c** (28.2 ± 2.8, p=0.44) showed decreased percentage of neurite-bearing cells but not reached the statistic significant when compared with Hp-s1, indicating that the α-conformation and orientation of C4-OH of glucose might have minor negative effects on the activity of Hp-s1 to induce neurite outgrowth. Notably, the percentage of neurite-bearing cells was significantly lower than the Hp-s1 if both the C4-OH and β-conformation were modified as compound **2d** (19.6 ± 3.1, p=0.01). Surprisingly, treatment of compound **2e** (20.4 ± 1.9, p=0.015) or **2f** (22.5 ± 3.81, p=0.042) led to a significant decrease in neurite outgrowth, suggesting that the C2-OH of glucose in Hp-s1 plays an important role in the stimulation of neuronal cells. Interestingly, percentage of neurite-bearing cell only

slightly decreased in **2a**-treated cells (28.4 ± 2, p=0.48) compared to Hp-s1-treated cells, implying that the sialic acid group might not be a pivotal functional group in Hp-s1 to provoke neurite formation. Additionally, compound **2a**-**2c** increased the percentage of neurite-bearing cells when compared with untreated and DMSO-treated cells, although they did not reach the statistically significant. Similar results were obtained for the percentage of branch point count (Figure 3B).

On the other hand, the structure of compound **2b** and **2d** is similar to α-galactosyl ceramide (α-GalCer) (Figure 1), which was originally extracted from marine sponge. α-GalCer is a well-known glycolipid, which could stimulate invariant natural kill T cells (NKT) to secrete potent cytokines to regulate immune response. To examine whether Hp-s1 analogues **2a**-**2f** could stimulate mouse NK1.2 cells, CD1d-expressing A20 cells were loaded with Hp-s1 **2a**-**2f** analogues (0.1 uM and 1 uM) and then incubated with mouse NK1.2 cells for three days. Supernatant was collected from the cultured cells and production of interleukin 2 (IL-2) was determined by ELISA. As shown in figure 4, only compound **2d** activated the mouse NK1.2 cells to produce IL-2. Surprisingly, the mouse NK1.2 cells were not activated by compound **2b**, which is only difference in the orientation of C4 hydroxyl group of galactosyl moiety when compared with compound **2d**. None of β-conformation compounds (**2a**, **2c, and 2f**) displayed the ability to stimulate the mouse NK1.2 cells, especially the **2c** which was changed **2d** from α-conformation to β-conformation, indicating that the α-conformation is important for compound **2d** to activate the NKT cells.

Conclusion

We have achieved the total synthesis of various Hp-s1 analogues **2a**-**2f** with replacing the glucosyl moiety of Hp-s1 with α-glucose, α- and β-galactose, α- and β-mannose. The effects of orientation of hydroxyl group at C2 and C4 position of glucosyl moiety of Hp-s1 on neurite outgrowth of SH-SY5Y cells and activation of NKT cells were investigated. We found that C2 hydroxyl group of glucosyl moiety in Hp-s1 plays an important role in the stimulation of neurite outgrowth of SH-SY5Y cells. In addition, the compound **2d** showed the ability to activate NKT cells, although it did not have any effect on neurite outgrowth of SH-SY5Y cells.

Materials and Methods

Neurite outgrowth and IL-2 production

Human neuroblastoma SH-SY5Y cells (ATCC CRL-2266) were obtained from American type cell culture (ATCC, Manassas, VA) and maintained in DMEM:Ham's F12 (1:1) medium supplemented with 10% fetal bovine serum at 37°C in 5% CO_2. For analysis of neurite outgrowth, cells (1×10^4) were seeded into each well of six-well plate and then incubated with indicated compounds (10 μM) for 72 hours. Neurite-bearing cells and neurite branch point were counted as described [17]. Triplicate wells for each tested compound and three random fields in each well were measured (40 cells/field). One-way analysis of variance (ANOVA) and Tukey's multiple comparison post hoc test was used for statistical analysis.

Mouse CD1d-overexpressing A20 cells were loaded with the indicated Hp-s1 analogues (0.1 and 1 μM) and then cultured with mNK1.2 cells. Three days later, supernatants were harvested and the level of IL-2 was determined by ELISA.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website.

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S. Y. Luo and A. L. Yu designed the research. J. T. Hung analyzed the data. C. H. Yeh, S. A. Yang and H. J. Tai prepared the compounds **2a**-**2f**. J. T. Hung wrote the manuscript with the help of G. B. Shelke and D. M. Reddy.

Abbreviations

Abbreviations are used as usual meanings.

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