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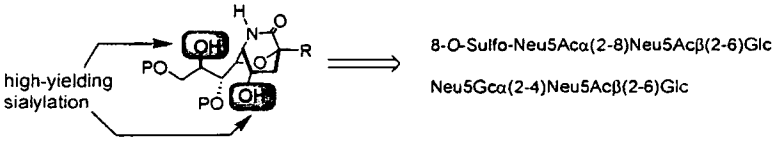
Sialylation

1,5-Lactamized Sialyl Acceptors for Various Disialoside Syntheses: Novel Method for the Synthesis of Glycan Portions of Hp-s6 and HLG-2 Gangliosides



A dramatic enhancement of the reactivity of the C4- and C8-hydroxy groups of sialic acid has been demonstrated by 1,5-lactam bridging. Sialyl-α(2→4)sialoside and sialyl-α(2→8)sialoside were made available in high yields through direct sialylation (see scheme). Furthermore, the glycan parts of the new gangliosides Hp-s6 and HLG-2 were synthesized for the first time.

H. Ando,* Y. Koike, S. Koizumi, H. Ishida, M. Kiso* _____ 6759 – 6763

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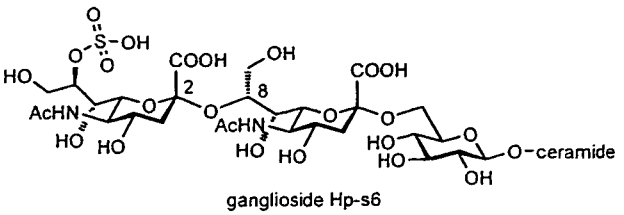
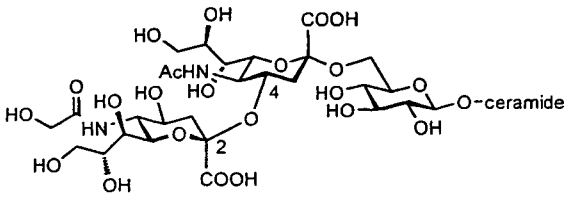
1,5-Lactamized Sialyl Acceptors for Various Disialoside Syntheses: Novel Method for the Synthesis of Glycan Portions of Hp-s6 and HLG-2 Gangliosides**

Hiromune Ando, Yusuke Koike, Sachiko Koizumi, Hideharu Ishida, and Makoto Kiso**

The ongoing studies on oligosaccharide synthesis have resulted in the development of precise synthetic methods by which a large portion of the complex natural oligosaccharides can be duplicated.[1] Although the synthesis of the sequence Neu5Acα(2→8)Neu5Ac (α(2→8)disialic acid; Neu5Ac = *N*-acetylneuraminic acid) has been a major difficulty, the emergence of several exquisite methods[2] that employ indirect coupling by using a C3-functionalized *N*-acetyl sialyl donor and direct coupling by using an *N*-trifluoroacetyl (TFAc)-protected sialic acid donor with the help of the nitrile solvent effect have paved the way for the successful synthesis of α(2→8)disialic acid containing oligosaccharides, such as those with GD3[2c] and GQ1b[3] glycan portions. However, it is obvious that the synthesis of new congeners of disialic acid, such as 8-*O*-sulfo-Neu5Acα(2→8)Neu5Ac in ganglioside Hp-s6[4] and Neu5Gcα(2→4)Neu5Ac in ganglioside HLG-2[5] (Scheme 1), is still difficult because of the diverse modifications possible at the functionality level. On the basis of the predicted biological functions of the disialic acid congener containing oligosaccharides relevant to functions such as neural network formation and fertilization, the establishment of an expedient synthetic method that includes the entire disialic acid family seems essential not only for the progress of glycochemistry but also for studying in detail the molecular basis underlying the biological functions of these compounds. In this study, we report a novel synthetic method for the synthesis of disialic acid congener containing glycans that uses highly reactive lactamized sialyl acceptors and an *N*-2,2,2-trichloroethoxycarbonyl (Troc)-protected sialyl donor.





Scheme 1. Structures of novel disialyl gangliosides Hp-s6 and HLG-2.

Recently, we reported an *N*-Troc-protected sialyl donor (*N*-Troc donor **1**) that shows elevated reactivity and a high degree of accessibility for various sialic acid congeners such as *N*-glycolylneuraminic acid (Neu5Gc), 8-*O*-sulfo-Neu5Ac, and 1,5-lactam-Neu.[6] Initially, we anticipated that use of the *N*-Troc donor would enable the design of HLG-2 and Hp-s6 glycan sequences in an expedient manner. However, as depicted in Scheme 2, the results of the condensations with 4-OH and 8-OH sialyl acceptors, **2** and **3**, respectively, did not meet expectations with regard to yields and stereoselectivity. Even in the case of **2**, which showed the relatively higher reactivity, α-disialyl glycoside was obtained in less than 5%. We hypothesized that the poor results were mainly due to unfavorable hydrogen bonding with the amide moiety at C5, as proposed previously by Tsvetkov and Schmidt.[7] This hypothesis was the basis of the idea that the conformational transformation from the $^2C_5$ chair form to the fixed boat form with the 1,5-lactam bridge would result in increased reactivity of both the C4- and C8-hydroxy groups.[8]

To form the 1,5-lactam bridge in the sialoside, the previously reported *N*-TFAc-sialic acid derivative **6**[9] was used as the key precursor (Scheme 3). After the coupling reaction of **6** and tribenzylated glucosyl acceptor **7**, the resulting sialyl-α(2→6)Glc disaccharide, **8**, was subjected to 1,5-lactamization. First, we attempted a carbodiimide-mediated intramolecular amide formation after the complete deacylation and saponification of **8**, but this reaction yielded a complex mixture. The optimum yield was obtained when **8** was treated with methanolic sodium methoxide in the presence of Drierite under reflux to provide the 1,5-lactam-sialyl glucoside **9** in 85% yield; through regioselective benzoylation of the C9-hydroxy group of **9** with benzoyl chloride and pyridine, under kinetic control, triol acceptor **10** was produced. For the synthesis of the 8-hydroxy-1,5-lacta-

[*] Dr. H. Ando
Division of Instrumental Analysis
Life Science Research Center
Gifu University
1-1 Yanagido, Gifu-shi, Gifu 501-1193 (Japan)
Fax: (+81) 58-293-2617
E-mail: hando@cc.gifu-u.ac.jp
Y. Koike, S. Koizumi, Dr. H. Ishida, Dr. M. Kiso
Department of Applied Bioorganic Chemistry
Faculty of Applied Biological Sciences
Gifu University
1-1 Yanagido, Gifu-shi, Gifu 501-1193 (Japan)
Fax: (+81) 58-293-2918
E-mail: kiso@cc.gifu-u.ac.jp

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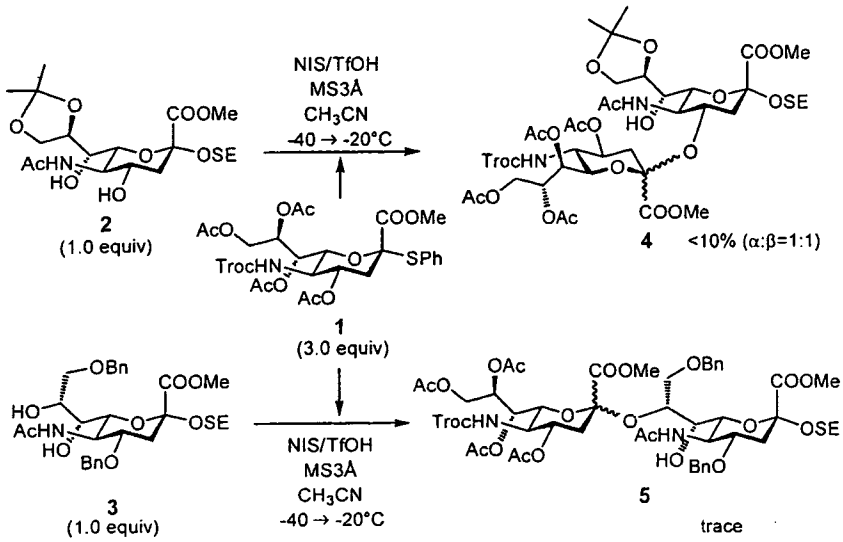
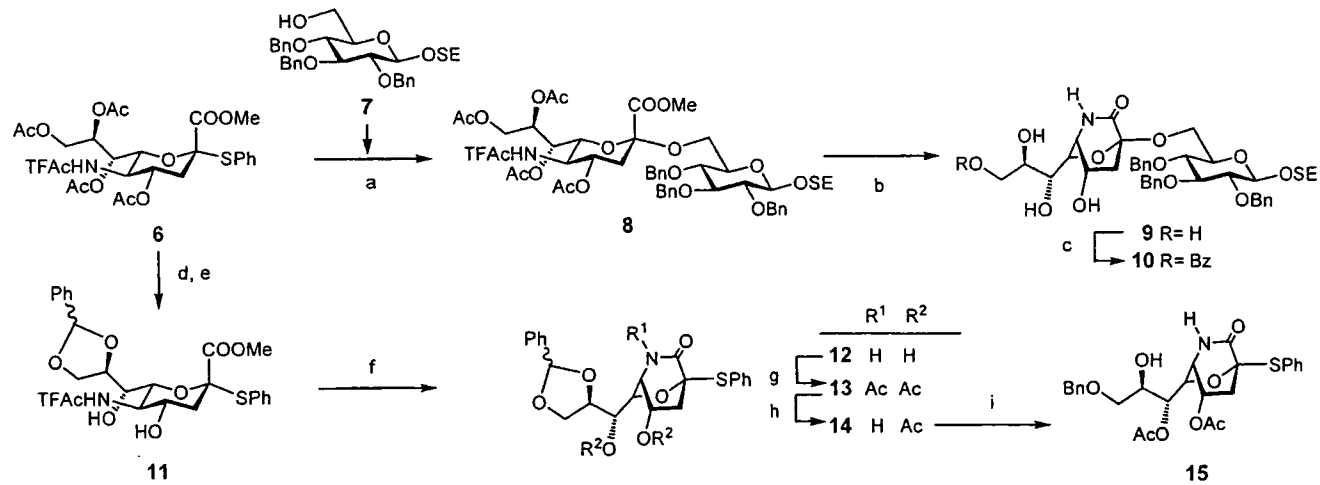


Scheme 2. Unsuccessful sialylation to 4-OH and 8-OH sialyl acceptors. Bn = benzyl, MS = molecular sieves, NIS = *N*-iodosuccinimide, SE = 2-(trimethylsilyl)ethyl, TfOH = trifluoromethanesulfonic acid.

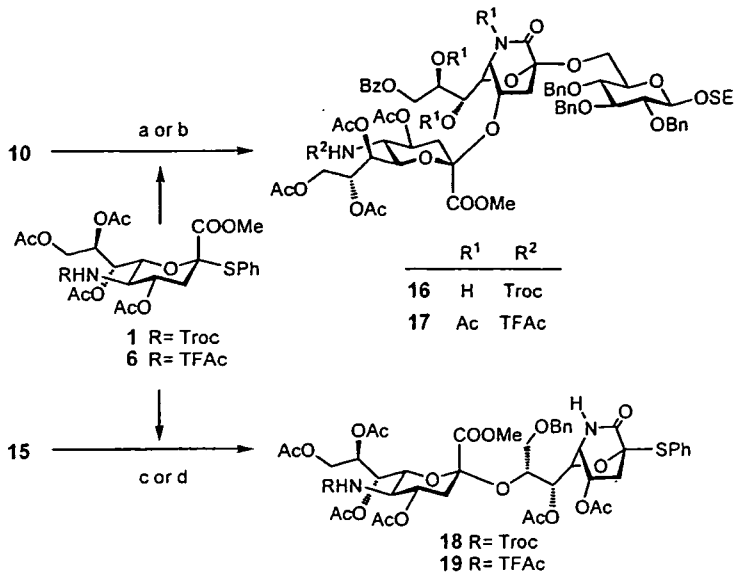
mized sialyl acceptor, 8,9-*O*-benzylidenation was required prior to the lactamization because 8,9-*O*-acetalization of the lactamized derivative was unsuccessful. Thus, compound **6** was de-*O*-acetylated and this was followed by the conventional 8,9-*O*-bezylidenation with benzaldehyde dimethyl acetal and camphorsulfonic acid to produce **11**, which was then subjected to the one-pot 1,5-lactam formation mentioned earlier to yield bicyclo-sialoside **12** in 89% yield. Next, **12** was completely acetylated with Ac_2O in the presence of pyridine and the product was successively de-*N*-acetylated with hydrazinium acetate in a chemoselective manner to produce compound **14**. Finally, reductive ring opening of the benzylidene acetal moiety, influenced by $BH_3{\cdot}NMe_3$ and $AlCl_3$ in THF,[10] produced the 8-OH lactam acceptor **15**.

Next, we carried out the glycosylation of the lactam acceptors **10** and **15** with *N*-Troc- and *N*-TFAc-sialyl donors to evaluate their properties as glycosyl acceptors (Scheme 4). First, the triol acceptor **10** was treated with *N*-Troc donor **1** in the presence of NIS, TfOH, and a molecular sieve in EtCN[11] at −40 °C to provide the Neuα(2→4)Neuα-(2→6)Glc sequence **17**, along with the corresponding β isomer. The anomeric configuration of the new ketosidic linkage was determined on the basis of previous reports[12] by measuring the long-range $^3J_{C1,H3ax}$ coupling constants. For compound **17** this coupling constant was 5.4 Hz, whereas for the β isomer it was less than 1.0 Hz, a fact indicating that the amomeric configuration of **17** was α. Similarly, the coupling reaction with the *N*-TFAc donor **6** and the complete acetylation that followed yielded the corresponding Neuα(2→4)Neuα(2→6)Glc sequence **17** in 41% yield, along with the β isomer (10%) and the Neu(2→8)[[Neu(2→4)]Neuα](2→6)Glc sequences as an anomeric mixture (8%).

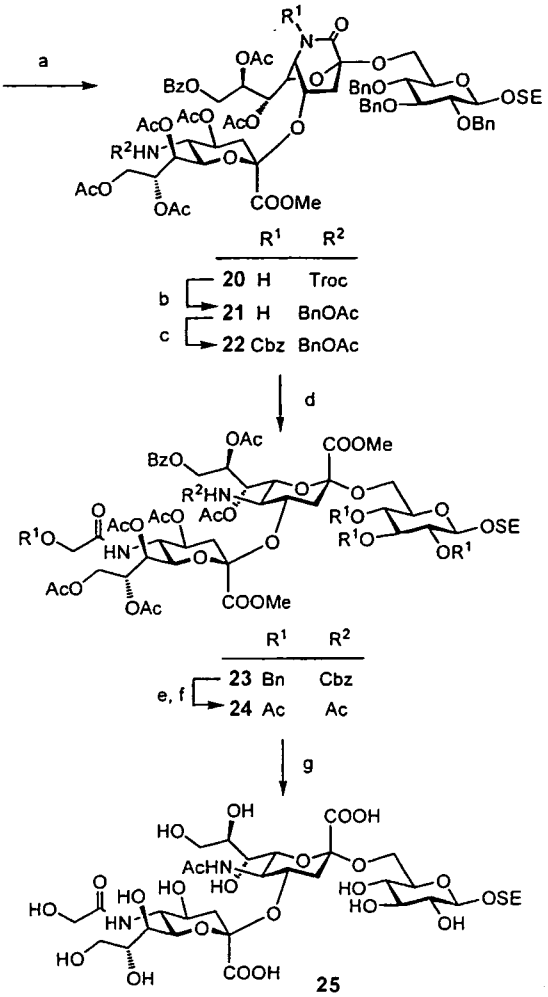
Next, we attempted to fashion the purest form of Neuα(2→8)Neu sequence (Scheme 4). As initially expected, the glycosylation reactions of the lactam acceptor **15** with *N*-Troc and *N*-TFAc donors (**1** and **6**) yielded the corresponding Neuα(2→8)Neu sequences. Thus, *N*-Troc donor **1** and *N*-TFAc donor **6** were incorporated, in the presence of NIS, TfOH, and a molecular sieve in EtCN, at −80 °C to yield α(2→8)disialosides **18** and **19** in 49 and 71% yield, respectively; no corresponding β form was generated in either event. To the best of our knowledge, the yield of addition to the C8-hydroxy group of sialic acid (71%) during the sialylation process was the highest value obtained by direct coupling methods[2] In keeping with the results of the previous experiments, the anomeric configuration of the new linkages was determined to be α from $^3J_{C1,H3ax}$ coupling constants that



Scheme 3. a) **7**, NIS, TfOH, CH_3CN/CH_2Cl_2, MS (3 Å), −30 °C, 5 min, 74%; b) NaOMe, MeOH, Drierite, reflux, 44 h, 85%; c) BzCl, py/CH_2Cl_2, −40 °C, 90 min, 79%; d) NaOMe, MeOH, room temperature, 29 h; e) $PhC(OMe)_2$, CSA, DMF, 40 °C, 2 h, 88% (2 steps); f) NaOMe, MeOH, Drierite, reflux, 5 d, 89%; g) Ac_2O, py, DMAP, room temperature, 3 h; h) $NH_2NH_2{\cdot}AcOH$, THF, room temperature, 80 min, 94% (2 steps); i) $BH_3{\cdot}NMe_3$, $AlCl_3$, THF, MS (4 Å), 0 °C → RT, 6 h, 74%. Bz = benzoyl, CSA = (±)-10-camphorsulfonic acid, DMF = *N*,*N*-dimethylformamide, DMAP = 4-dimethylaminopyridine, py = pyridine, THF = tetrahydrofuran.



Scheme 4. a) **1** (2.0 equiv), NIS (3.0 equiv), TfOH (0.3 equiv), EtCN, MS (3 Å), −40 °C, 6 h, 84% (α/β 66:18); b) 1. **6** (2.0 equiv), NIS, TfOH, EtCN, MS (3 Å), −40 °C, 6 h; 2. Ac_2O, py, DMAP, 40 °C, 17 h, 51%; c) **1** (3.0 equiv), NIS, TfOH, EtCN, MS (3 Å), −80 °C, 5 h, 49% (α only); d) **6** (3.0 equiv), NIS, TfOH, EtCN, MS (3 Å), −80 °C, 3 h, 71% (α only).



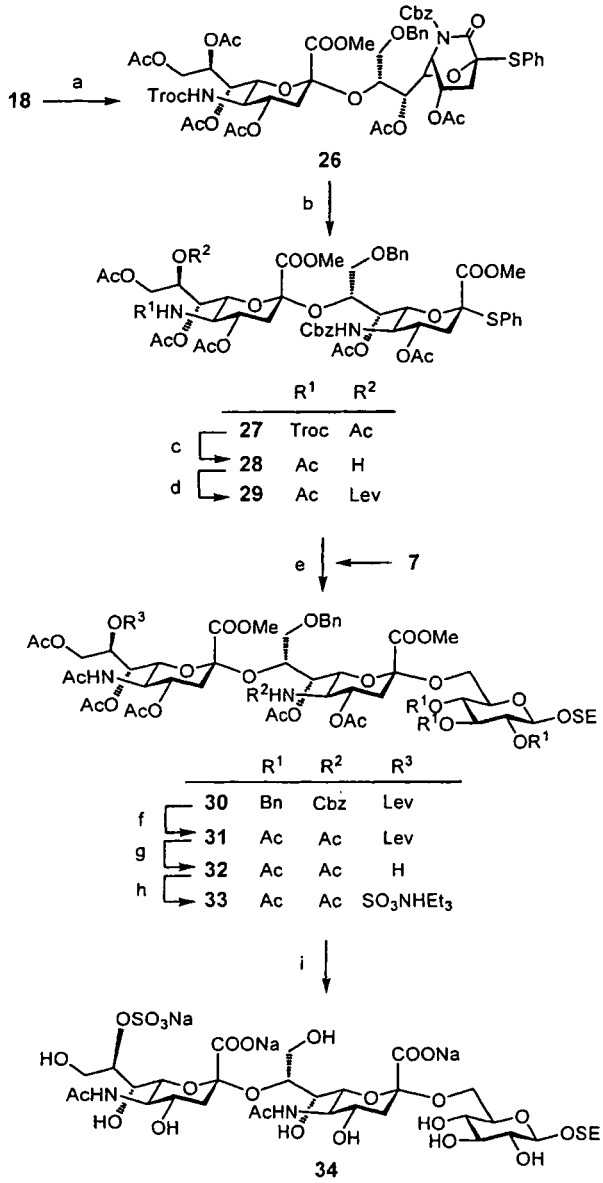
Scheme 5. a) Ac_2O, py, room temperature, 10 h, 89%; b) 1. Zn, AcOH, room temperature, 2 h; 2. BnOAcCl, THF, room temperature, 1 h, 74% (2 steps); c) Cbz_2O, DMAP, py, 40 °C, 26 h, 95%; d) 1. Et_3N, H_2O/CH_3CN, room temperature, 2 d; 2. MeI, K_2CO_3, DMF, room temperature, 30 min, 74% (2 steps); e) 1. H_2, 10% $Pd(OH)_2$/C, NH_3, EtOH, room temperature, 2 h; 2. AcCl, room temperature, 1 h, 68% (2 steps); f) 1. H_2, 10% $Pd(OH)_2$/C, EtOH; 2. Ac_2O, py, room temperature, 54% (2 steps). Cbz = benzyloxycarbonyl.

ranged from 6.7 to 6.9 Hz. Furthermore, the phenylsulfenyl group at the bridgehead anomeric center of acceptor **15** remained unaffected during the coupling reactions. This result confirmed our initial hypothesis, based on Bredt's rule, suggesting the basis of a novel method for the complete deactivation of a sialyl donor.

On the basis of the results obtained with regard to the performance of 1,5-lactamized sialic acid acceptors **10** and **15** in the sialylation reactions, we focused on the synthesis of the glycan portions of HLG-2 and Hp-s6 gangliosides of **16** and **18**, respectively, in order to demonstrate the practical efficacy of the synergic strategy for synthesizing variant disialosides from the 1,5-lactam-sialyl acceptor and *N*-Troc-sialyl donor.

In the initial stages of the synthesis of the HLG-2 glycan portion (Scheme 5), the trisaccharide **16** was *O*-acetylated to provide **20**, to which the *N*-glycolyl moiety was introduced by our reported method,[6] thereby providing **21** in a relatively high yield (66% from **16**). Next, we attempted to recover the $^2C_5$ conformation of the inner sialic acid unit. The following reaction sequences supplied HLG-2 glycan frame **23** in a high yield: *N*-benzyloxycarbonylation, basic hydrolysis, and ensuing methylation of the carboxy group. Debenzylation and acetylation to replaced the Cbz group of **23** by the acetyl group and full deprotection of the product **24** yielded the HLG-2 glycan structure **25**.

In the case of the Hp-s6 glycan frame, the "locked-up" phenylsulfenyl group at the bridgehead carbon atom of **18** was converted into an active state in the initial stages (Scheme 6). To be precise, the reaction sequences mentioned earlier yielded $^2C_5$ conformer **27** in 62% overall yield. For the purpose of 8-*O*-sulfonylation in the final stages of the synthesis, **27** was further transformed into the 8-hydroxy derivative **28** by our regioselective acetyl-transfer method,[6] and the C8 hydroxy group was capped with a levulinoyl group to produce high yields (89%) of the suitably protected disialic acid donor **29** in two steps. Compound **29** was then treated with glucosyl acceptor **7**, influenced by the NIS/TfOH activator system in EtCN at −80 → −60 °C, to provide Neuα-(2→8)Neuα(2→6)Glc sequence **30** in 66% yield, predominantly in the α configuration. Next, replacement of the Cbz and benzyl groups of trisaccharide **30** by the acetyl group, followed by chemoselective deblocking of the levulinoyl group with hydrazinium acetate[13] and sulfonylation with SO_3·pyridine resulted in the formation of a completely protected Hp-s6 glycan frame, **33**.[14] The ^{1}H NMR signal for the C8 proton of the outer sialic acid appeared in compound **33** at lower magnetic field ($\delta = 4.92$ ppm) than in compound **32** ($\delta = 4.22$ ppm), and the heteronuclear multiple-bond coherence (HMBC) spectrum of compound **33** contained cross-coupling signals between carbonyl carbon atoms of acetyl groups at C7 and C9, and H7 ($\delta = 5.40$ ppm) and H9



Scheme 6. a) CbzOSu, DMAP, py, room temperature, 42 h, 79%; b) 1. Et_3N, H_2O/CH_3CN, 40°C, 45 h; 2. MeI, K_2CO_3, DMF, room temperature, 3 h, 79% (2 steps); c) Zn, AcOH, THF, room temperature, 28 h, 94%; d) LevOH, DCC, DMAP, CH_2Cl_2, room temperature, 2 h, 95%; e) **7**, NIS, TfOH, EtCN, MS (3 Å), −80→−60°C, 4 d, 66%; f) 1. H_2, 10% $Pd(OH)_2/C$, NH_3, EtOH, room temperature, 1 h; 2. Ac_2O, py, room temperature, 30 min; 3. H_2, 10% $Pd(OH)_2/C$, EtOH, 40°C, 3 h; 4. Ac_2O, py, room temperature, 12 h, 86% (4 steps); g) NH_2NH_2·AcOH, EtOH, room temperature, 6 h, 90%; h) SO_3·py, py, room temperature, 7 h, 65%. Lev = levulinoyl = 4-oxopentanoyl, Su = succinimidyl, DCC = *N*,*N*′-dicyclohexyl carbodiimide.

($\delta = 4.19$ ppm). Thereby, the installation of the sulfonyl group on the C8-hydroxy group was determined.

In conclusion, we have discovered that 1,5-lactam bridging in sialic acid endows high reactivity to the C4- and C8-hydroxy groups, thereby leading to the supply of α(2→4)- and α(2→8)disialic acid sequences in high yields. Furthermore, the practical efficacy of the synergic synthetic approach toward diverse disialic acid containing oligosaccharides, based on the *N*-Troc donor and the lactamized acceptors as the main units, has been demonstrated by the novel method for the synthesis of the HLG-2 and Hp-s6 glycan chains. On the basis of these results, we are now investigating the synthesis of α(2→8)-linked oligosialic acids.

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Keywords: gangliosides · glycosylation · lactams · sialic acids

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Design and synthesis of versatile ganglioside probes for carbohydrate microarrays

Akihiro Imamura · Takeru Yoshikawa · Tatsuya Komori · Masatoshi Ando · Hiromune Ando · Masahiro Wakao · Yasuo Suda · Hideharu Ishida · Makoto Kiso

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Abstract A series of ganglioside GM1-, GM2-, and GM3-type probes, in which the ceramide portion is replaced with a glucose residue, were systematically synthesized based on a convergent synthetic method.

Keywords Chemical synthesis · Gangliosides · Glycosylation · Carbohydrate probe

Introduction

Gangliosides, anionic glycosphingolipids with various sugar chains containing one or more residues of sialic acid, exist universally on cell surface. They participate in vital processes, such as immune or nervous systems, as molecules responsible for cell–cell and cell–ligand interactions [1, 2]. In particular, a series of gangliosides, such as GM1, GM2 and GM3, are important as regulatory factors for the differentiation of the central nervous system and serve as cell-attachment receptors for some viruses, bacteria and bacterial toxins [3, 4]. Moreover, many profound relationships between those gangliosides and a number of cancers and diseases have been demonstrated [5, 6]. However, the biological functions of gangliosides are not fully understood, due to their structural complexities and the low affinities of interaction with ligands, despite numerous studies conducted to date. To solve these issues, a considerable number of efforts have gone into the development of analytical techniques for sensitive detection of carbohydrate–ligand interactions. Consequently, many carbohydrate microarray technologies have been developed to facilitate glycomics research [7]. Coincidentally, many carbohydrate probes that incorporate specific functional groups such as azide [8], thiol [9] and maleimide [10] have been chemically synthesized for the fabrication of microarrays. Recently, oligosaccharide-immobilized chips (named Sugar_Chips), which provide real-time and high-throughput analysis of oligosaccharide–protein interaction without any labeling of the targeted protein, have been developed [11], in which chemically synthesized oligosaccharides having D-glucose, which provides a reactive aldehyde functionality, at the reducing end were used. The D-glucose residue also serves as a spacer between a targeted sugar chain and a scaffold for immobilization, because of its appropriate hydrophilicity and flexibility. Furthermore, it has been demonstrated that a reducing sugar directly participates in the noncovalent link to a scaffold [12, 13]. Accordingly, as exemplified in Fig. 1, the chemically synthesized oligo-

A. Imamura (✉) · T. Yoshikawa · T. Komori · M. Ando · H. Ishida · M. Kiso (✉)
Department of Applied Bioorganic Chemistry, Faculty of Applied Biological Sciences, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan
e-mail: gif012@gifu-u.ac.jp
e-mail: kiso@gifu-u.ac.jp

H. Ando
Division of Instrumental Analysis, Life Science Research Center, Gifu University, 1-1 Yanagido, Gifu-shi, Gifu 501-1193, Japan

M. Wakao · Y. Suda
Department of Nanostructure and Advanced Materials, Graduate School of Science and Engineering and Venture Business Laboratory, Kagoshima University, Kohrimoto, Kagoshima 890-0065, Japan

M. Kiso
Institute for Integrated Cell-Material Sciences (iCeMS), Kyoto University, Kyoto, Japan

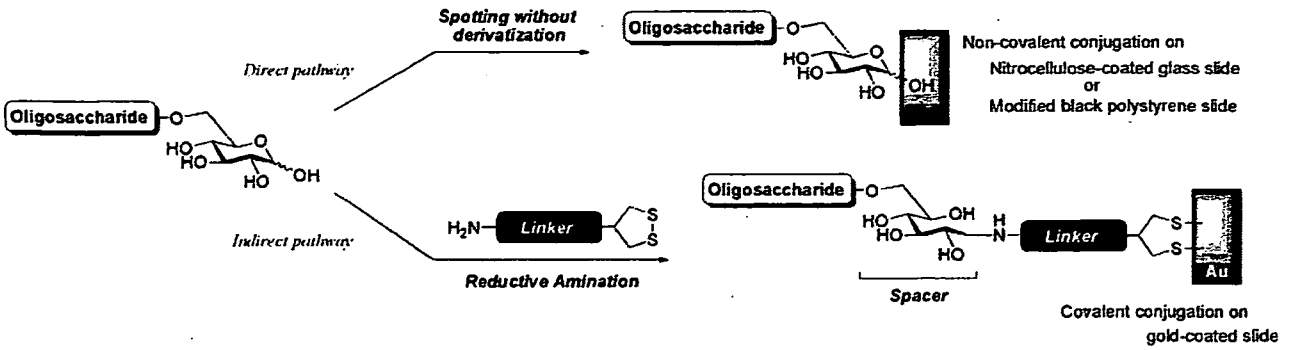


Fig. 1 Two examples for carbohydrate microarray fabrication

saccharide probes are expected to be immobilized by the direct and indirect attachment to scaffolds. We report here the facile synthesis of glucose-ended probes of ganglioside GM1, GM2, and GM3 for carbohydrate microarrays (Fig. 2).

Results and discussion

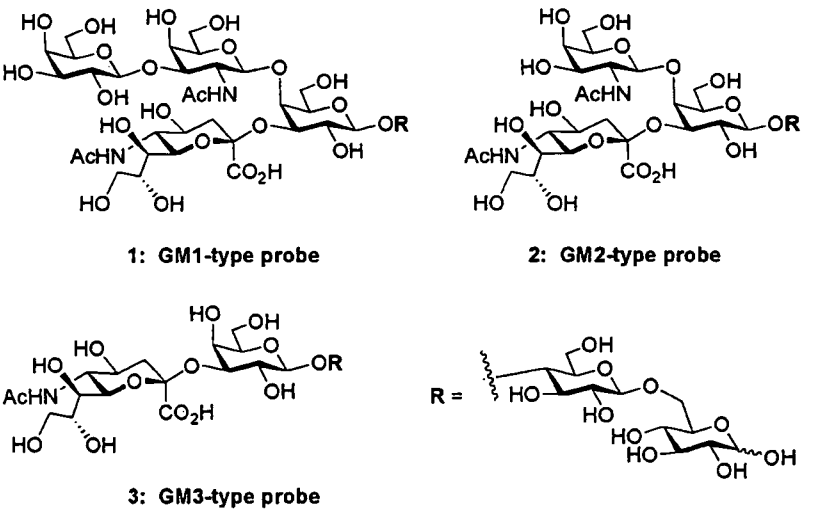
Taking a look at target molecules, we have hypothetically disconnected them into two parts: common sequence SAα(2→3)Galβ(1→4)Glcβ(1→6)Glc, and the other sugar parts. The common sequence was further disconnected at Galβ(1→4)Glc linkage, providing SAα(2→3)Gal and gentiobiose segments, based on the recently reported efficient syntheses of GM2 analogs [14]. Considering the difficulty to fashion a branch out from galactose residue, the incorporation of GalN parts into Gal residue was planned to be conducted earlier than that of gentiobiose as depicted in Fig. 3.

According to our previous report [14], 2,6-*O*-dibenzylated galactoside was efficiently sialylated at C-3 position with *N*-Troc-protected sialyl donor [15, 16], producing a key sialyl galactoside **4**, which can be obtained in a crystalline form after rough chromatographic purification of the reaction mixture (Fig. 4).

The disaccharide **4** was coupled with Galβ(1→3)GalN **6** [17] or GalN donor **5** in the presence of NIS and TfOH [18] to afford the GM2-core trisaccharide **7** in 97% yield and the GM1-core tetrasaccharide **8** in 89% yield, respectively, as depicted in Table 1.

A series of ganglioside-core frames **4**, **7**, and **8** were converted into the corresponding glycosyl donors **13**, **14**, and **15**, respectively. The selective removal of the Troc group of **4** by the action of zinc–copper couple [19, 20] in acetic acid/1,2-dichloroethane at 40°C proceeded smoothly to give a free amino derivative, which, on successive treatment with acetic anhydride in pyridine afforded the corresponding *N*-acetyl derivative **9**. The use of 1,2-dichloroethane (DCE) was critical for an efficient reduction of Troc group; otherwise the reaction was sluggish. Initially, we were afraid that DCE as solvent itself consumes zinc–copper couple as reductant. Though it is not clear whether DCE is advantageous for electron transfer from zinc–copper couple, we were intriguingly able to observe smooth proceeding of the reaction in a single liquid

Fig. 2 Structure of synthetic ganglioside probes



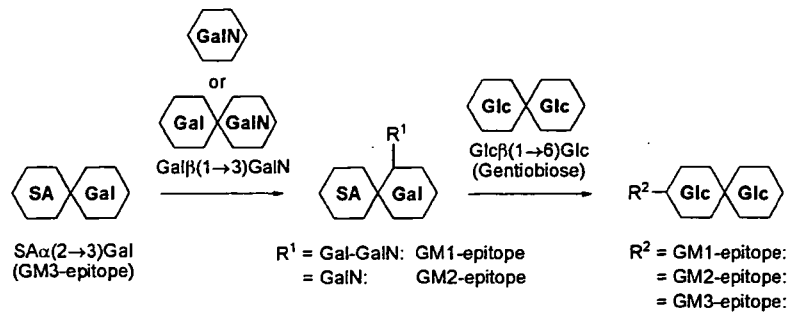


Fig. 3 Systematic reaction scheme for preparation of the reductive glucose-functionalized ganglioside probes

phase within a short time. The cleavage of benzyl groups was executed by hydrogenolysis and the following benzoylation of the resulting hydroxyl groups gave **11**. Libration of the anomeric hydroxyl group of **11** was achieved by treatment with ceric ammonium nitrate (CAN) in acetonitrile–toluene–water (6:5:3) [21]. The obtained hemiacetal was then converted into the β-trichloroacetimidate **13**, which was ready for the final glycosylation with the gentiobiose acceptor **21** as mentioned hereinafter. Interestingly, the use of less than a stoichiometric amount of DBU resulted in the predominant formation of the β-imidate derivative. The conversion of **7** and **8** into the corresponding donor **14** and **15** were also achieved by similar procedure, respectively. (Scheme 1)

Scheme 2 shows the preparation of the gentiobiose acceptor **21** as the common synthetic block, which was expected to have an enhanced reactivity at C-4 hydroxyl due to the effect of electron-donating benzyl groups. Coupling of the known glucose donor **16** [22] and acceptor **17** [23] was conducted in the presence of NIS and TfOH in CH_2Cl_2 at 0°C to give the disaccharide **18** in 90% yield. The β-configuration of the newly formed intersaccharide linkage between **16** and **17** is apparent from the relatively large coupling constant (8.2 Hz) between H-1′ and H-2′ in ^{1}H NMR spectra. Removal of the benzoyl groups under conventional conditions and benzylation of the hydroxyl groups gave **20** with a yield of 88% in two steps. Finally, reductive opening of the benzylidene group was achieved by a treatment with triethylsilane and $BF_3{\cdot}OEt_2$ in CH_2Cl_2 [24] to afford **21** with a yield of 85%.

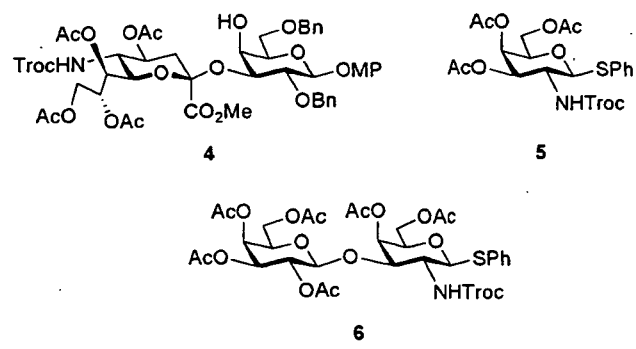


Fig. 4 Structure of glycosyl acceptor (**4**) and donors (**5**, **6**)

Scheme 3 incorporates final glycosylations of **21** with a series of ganglioside-core donors, **13**, **14**, and **15** in the presence of TMSOTf in CH_2Cl_2 at 0°C. The β-imidate **13** was coupled with the gentiobiose acceptor **21** by treatment with TMSOTf at 0°C to afford the desired β-glycoside **22** in an excellent yield. The α-imidate **14** and **15** were subjected to the glycosidation with **21** under essentially the same conditions for **13** to give **23** and **24** in good yields, respectively. Finally, global deprotection of the above-mentioned glycans was conducted. After de-acylation under Zemplén conditions and subsequent saponification of the fully protected oligosaccharides, **24**, **23**, and **22**, hydrogenolysis for each resultant compound was performed in the presence of $Pd(OH)_2/C$ under H_2 atmosphere to afford the target carbohydrate probes **1**, **2** and **3** in good to excellent yields, respectively.

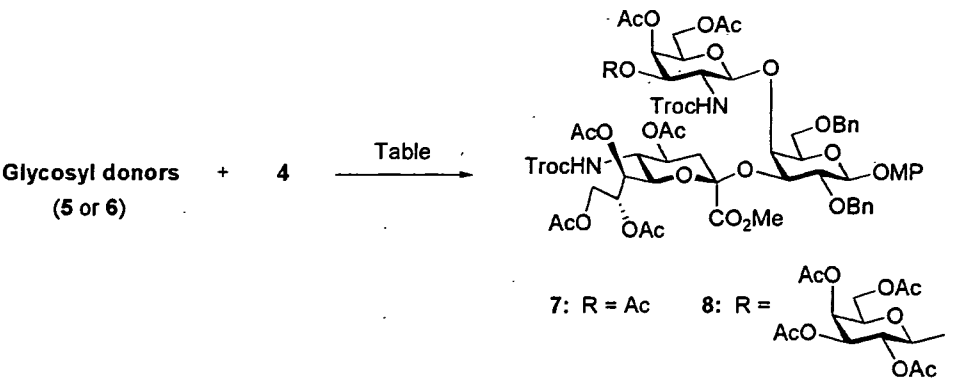
In conclusion, we have succeeded in the synthesis of ganglioside GM1-, GM2-, and GM3-type probes for carbohydrate microarray analyses. It was found that the convergent synthetic strategy between the defined ganglioside-core frame and the reducing end glucose can be used for the synthesis of complex ganglioside probes. In addition, synthesized ganglioside probes are currently used as one of the oligosaccharide probes on immobilized-chips by Suda's group. We are currently underway to expand the existing pool of functional carbohydrate probes containing more complex gangliosides.

Experimental

General procedures

All reactions were carried out under a positive pressure of argon, unless otherwise noted. All chemicals were purchased from commercial suppliers and used without further purification, unless otherwise noted. Molecular sieves were purchased from Wako Chemicals Inc. and dried at 300°C for 2 h in muffle furnace prior to use. ^{1}H NMR and ^{13}C NMR spectra were recorded with a Varian Inova 400/500 spectrometer and a JEOL ECA 500/600 spectrometer. Chemical shifts are reported in parts per million (ppm) downfield from tetramethylsilane. Data are presented as

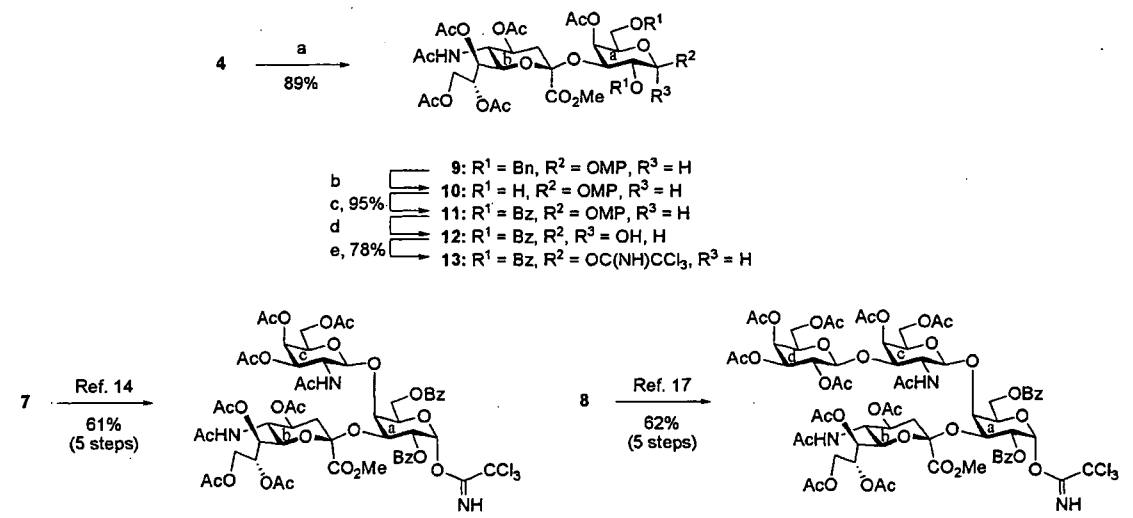
Table 1 Glycosylation of **4** with glycosyl donors **5** and **6**



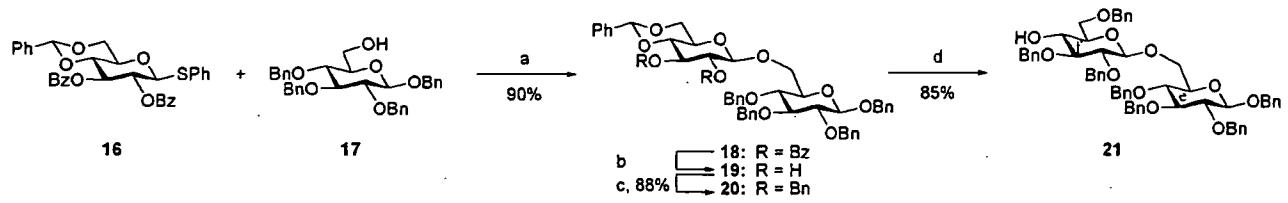
Entry	Donor	Condition	Temp.[°C]	Product	% Yield
1	**5**	NIS TfOH MS4Å CH_2Cl_2	0	**7**	97
2	**6**		-40	**8**	89

follows: Chemical shift, multiplicity (s=singlet, d=doublet, t=triplet, dd=double of doublet, m=multiplet and/or multiple resonances), integration, coupling constant in Hertz (Hz). MALDI-TOF MS spectra were recorded in the positive ion mode on a Bruker Autoflex with the use of α-cyano-4-hydroxy-cinnamic acid (CHCA) as a matrix. Optical rotations were measured with a 'Horiba SEPA-300' polarimeter. Column chromatography was performed on silica gel (Fuji Silysia Co., 80 and 300 mesh). Reactions were monitored by TLC on silica gel $60F_{254}$ (Merck, glass plate) and the compounds were detected by examination under UV light (2,536 Å) and visualized by dipping the plates in a 10% sulfuric acid–ethanol solution or 20% phosphomolybdic acid–ethanol solution followed by heating. Organic solutions were concentrated by rotary evaporation below 45°C under reduced pressure. Solvent systems in chromatography were specified in *v/v*.

4-Methoxyphenyl {methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-4-O-acetyl-2,6-di-O-benzyl-β-D-galactopyranoside (**9**) To a solution of compound **4** (500 mg, 465 μmol) in 1,2-dichloroethane (6.1 ml) were added acetic acid (18.3 ml) and zinc–copper couple (2.50 g). The mixture was stirred for 1.5 h at 40°C, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=15:1). The



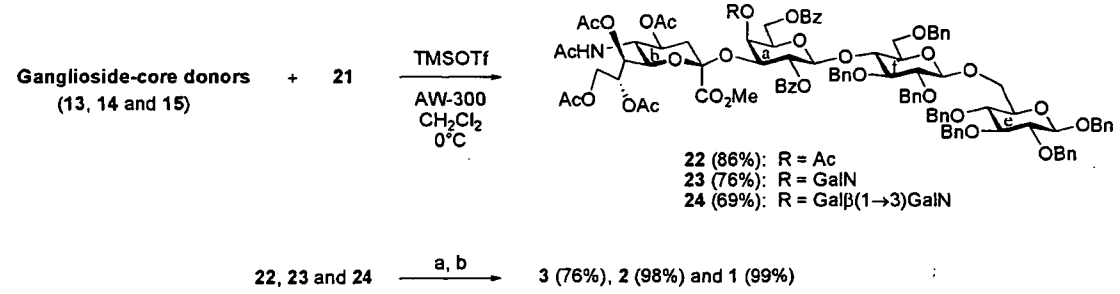
Scheme 1 Conversion of ganglioside-core frames to the corresponding glycosyl donors. Reagents and conditions: *a* Zn-Cu, AcOH, 1,2-DCE, 40°C then Ac_2O, Py; *b* $Pd(OH)_2/C$, H_2, EtOH; *c* Bz_2O, Py; *d* CAN, CH_3CN-PhMe-H_2O (6/5/3); *e* CCl_3CN, DBU, CH_2Cl_2, 0°C



Scheme 2 Preparation of the gentiobiosyl acceptor **21**. Reagents and conditions: *a* NIS, TfOH, MS4Å, CH_2Cl_2, 0°C; *b* NaOMe, MeOH-THF (2/1); *c* BnBr, NaH, DMF; *d* TESH, $BF_3{\cdot}OEt_2$, CH_2Cl_2

reaction mixture was filtered through Celite. The combined filtrate and washings was extracted with $CHCl_3$, and the organic layer was washed with H_2O, sat. Na_2CO_3, and brine, dried over Na_2SO_4 and concentrated. To a solution of the residue in pyridine (5.0 ml) was added acetic anhydride (2.5 ml). The mixture was stirred for 13 h at ambient temperature, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=15:1). The reaction mixture was coevaporated with toluene and extracted with $CHCl_3$. The organic layer was washed with 2 M HCl, H_2O, sat. $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel (EtOAc/hexane=3:1) to give **9** (406 mg, 89%).; $[\alpha]_D$=−15.4° (*c* 0.9, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.45–6.77 (m, 14 H, 2 Ph and 1 MP), 5.53 (m, 1 H, H-8b), 5.33 (dd, 1 H, H-7b), 5.24 (d, 1 H, $J_{5,NH}$=8.9 Hz, NH), 5.07 (m, 2 H, H-1a, 4a), 4.96–4.88 (m, 3 H, H-4b, 2 CH*H*Ph), 4.63 (dd, 1 H, H-3a), 4.53 (d, 1 H, CH*H*Ph), 4.46 (d, 1 H, CH*H*Ph) 4.36 (dd, 1 H, H-9'b), 4.13 (q, 1 H, $J_{5,NH}$= 8.9 Hz, H-5b), 3.96–3.94 (m, 2 H, H-6'a, 9b), 3.85 (s, 3 H, OMe), 3.76–3.73 (m, 5 H, H-2a, 6b, OMe), 3.56–3.52 (m, 2 H, H-5a, 6a), 2.63 (dd, 1 H, H-3b$_{eq}$), 2.12–1.83 (m, 19 H,6 Ac, H-3b$_{ax}$); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 170.9, 170.6, 170.3, 170.2, 170.0, 168.1, 155.1, 151.7, 139.4, 138.0, 128.3, 128.1, 127.7, 127.6, 127.1, 118.2, 114.4, 102.4, 97.1, 78.1, 74.8, 73.5, 73.1, 72.3, 72.2, 69.5, 68.9, 68.7, 68.6, 67.2, 62.2, 55.6, 53.1, 49.2, 37.6, 23.2, 21.3, 20.8, 20.8, 20.5; MALDI MS: *m/z*: calcd for $C_{49}H_{59}O_{20}NNa$: 1,004.35; found: 1,004.35 [*M* + Na] $^+$.

4-Methoxyphenyl {methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-4-O-acetyl-2,6-di-O-benzoyl-β-D-galactopyranoside (**11**) To a solution of compound **9** (385 mg, 392 μmol) in EtOH (30 ml) was added palladium hydroxide [$Pd(OH)_2$] (20 wt% Pd on carbon; 400 mg). The mixture was vigorously stirred for 4 h at ambient temperature under hydrogen atmosphere, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=15:1). The reaction mixture was filtered through Celite. The combined filtrate and washings was concentrated. To a solution of the residue in pyridine (5.0 ml) was added benzoic anhydride (354 mg, 1.57 mmol). The mixture was stirred for 16 h at ambient temperature, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=15:1). The reaction mixture was coevaporated with toluene and extracted with $CHCl_3$. The organic layer was washed with 2 M HCl, H_2O, sat. $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel (EtOAc/hexane=3:1) to give **11** (380 mg, 95%); $[\alpha]_D$=+ 27.9° (*c* 4.2, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.17–6.67 (m, 14 H, 2 Ph and 1 MP), 5.59 (m, 1 H, H-8b), 5.55 (t, 1 H, $J_{1,2}$=8.3 Hz, $J_{2,3}$=10.3 Hz, H-2a), 5.26 (d, 1 H, $J_{1,2}$=8.3 Hz, H-1a), 5.20 (dd, 1 H, $J_{6,7}$=2.8 Hz, H-7b), 5.16 (d, 1 H, $J_{3,4}$=3.4 Hz, H-4a), 5.14 (d, 1 H, NH), 4.87 (dd, 1 H, $J_{2,3}$=10.3 Hz, $J_{3,4}$=3.4 Hz, H-3a), 4.85 (m, 1 H, H-4b), 4.46 (t, 1 H, H-6'a), 4.35 (dd, 1 H, H-6a), 4.27 (dd, 1 H, H-9'b), 4.19 (t, 1 H, H-5b), 3.91 (dd, 1 H, H-9b), 3.86–3.79 (m, 4 H, H-5b, OMe) 3.71 (s, 3 H, OMe), 3.61 (dd, 1 H, $J_{6,7}$=2.8 Hz,



Scheme 3 Coupling of the ganglioside-core donors (**13**, **14** and **15**) and the gentiobioside acceptor (**21**), and subsequent global deprotections. Reagents and conditions: *a* NaOMe, MeOH, 45°C or reflux, then H_2O; (b) $Pd(OH)_2$/C, H_2, H_2O or MeOH-H_2O (5/2), RT or 40°C

H-6b), 2.59 (dd, 1 H, H-3b$_{eq}$), 2.19–1.44 (m, 19 H, 6 Ac, H-3b$_{ax}$); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 170.7, 170.6, 170.3, 170.2, 170.0, 168.0, 165.7, 165.3, 155.4, 151.3, 133.2, 133.0, 130.1, 130.0, 129.7, 128.3, 128.3, 118.9, 114.2, 101.1, 96.7, 71.6, 71.1, 70.8, 69.3, 67.6, 67.4, 66.4, 62.3, 62.0, 55.4, 53.0, 48.7, 37.2, 23.2, 21.3, 20.7, 20.1; MALDI MS: *m/z*: calcd for $C_{49}H_{55}O_{22}NNa$: 1,032.31; found: 1,032.38 [*M* + Na] $^+$.

{Methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-4-O-acetyl-2,6-di-O-benzoyl-β-D-galactopyranosyl Trichloroacetimidate (**13**) To a solution of compound **11** (164 mg, 162 μmol) in mixed solvent (MeCN/PhMe/H_2O= 3.5:2.9:1.7 ml) was added diammonium cerium(IV) nitrate (CAN; 445 mg, 812 μmol). The mixture was stirred for 5 h at ambient temperature, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=20:1). The reaction mixture was extracted with $CHCl_3$, and the organic layer was washed with H_2O, sat. $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH= 65:1) to give **12** (147 mg). To a solution of compound 12 in CH_2Cl_2 (5.0 ml) were added trichloroacetonitrile (410 μl, 407 μmol) and 1,8-diazabicyclo[5.4.0]-7-undecene (DBU; 4.9 μl, 33.0 μmol). The mixture was stirred for 2 h at 0°C, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=20:1). The reaction mixture was concentrated and the residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH=75:1) to give **13** (132 mg, 78%).; $[\alpha]_D$=+ 18.6° (*c* 0.8, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.67 (s, 1 H, C=NH), 8.10–7.41 (m, 10 H, 2 Ph), 6.20 (d, 1 H, $J_{1,2}$=8.3 Hz, H-1a), 5.60–5.56 (m, 2 H, H-2a, H-8b), 5.22–5.20 (m, 2 H, H-4a, H-7b), 4.98 (d, 1 H, $J_{5,NH}$=10.3 Hz, NH-b), 4.93 (dd, 1 H, H-3a), 4.87 (m, 1 H, H-4b), 4.49 (q, 1 H, H-6'a), 4.34–4.29 (m, 3 H, H-5a, 6a, 9'b), 3.93 (dd, 1 H, H-9b), 3.85–3.77 (m, 4 H, H-5b, OMe), 3.60 (dd, 1 H, H-6b), 2.58 (dd, 1 H, H-3b$_{eq}$), 2.19–1.43 (m, 19 H, 6 Ac, H-3b$_{ax}$); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 170.8, 170.7, 170.6, 170.2, 170.2, 170.0, 168.0, 165.7, 165.1, 161.1, 133.2, 130.1, 129.9, 129.7, 129.7, 128.3, 128.3, 96.8, 96.4, 90.3, 77.2, 71.8, 71.5, 71.1, 70.0, 69.4, 67.6, 67.4, 66.5, 62.4, 61.5, 53.1, 48.8, 37.3, 29.7, 23.1, 21.4, 20.8, 20.7, 20.2; MALDI MS: *m/z*: calcd for $C_{44}H_{49}O_{21}N_2Cl_3Na$: 1,069.18; found: 1,069.41 [*M* + Na] $^+$.

Benzyl 2,3-di-O-benzoyl-4,6-O-benzylidene-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**18**) To a solution of compound **16** (970 mg, 1.70 mmol) and **17** (762 mg, 1.41 mmol) in CH_2Cl_2 (31 ml) was added molecular sieves 4 Å (1.70 g). The suspension was stirred for 2 h and cooled to 0°C. To the mixture were added *N*-iodosuccinimide (NIS; 765 mg, 3.40 mmol) and trifluoromethanesulfonic acid (TfOH) (30 μl, 0.34 mmol) and stirring was continued for 1.5 h. Completion of the reaction was confirmed by TLC (EtOAc/hexane=1:3). The reaction mixture was filtered through Celite. The combined filtrate and washings was extracted with $CHCl_3$, and the organic layer was washed with sat. Na_2CO_3, sat. $Na_2S_2O_3$ and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel (EtOAc/hexane=1:5) to give **18** (1.26 g, 90%).; $[\alpha]_D$=-9.3° (*c* 1.0, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.95–7.13 (m, 35 H, 7 Ph), 5.75 (t, 1 H, $J_{2,3}$=8.8 Hz, $J_{3,4}$=8.6 Hz, H-3f), 5.55 (s, 1 H, >C*H*Ph), 5.52 (t, 1 H, $J_{1,2}$=8.2 Hz, $J_{2,3}$=8.8 Hz, H-2f), 4.91–4.86 (m, 3 H, H-1f, 2 CH*H*Ph), 4.77–4.65 (m, 4 H, 4 CH*H*Ph), 4.49–4.39 (m, 4 H, H-1e, 6f, 2 CH*H*Ph), 4.14 (d, 1 H, J_{gem}= 11.0 Hz, H-6e), 3.99 (t, 1 H, $J_{3,4}$=8.6 Hz, $J_{4,5}$=9.6 Hz, H-4f), 3.89 (br t, 1 H, J_{gem}=10.3 Hz, $J_{5,6}$=9.3 Hz, H-6'f), 3.73–3.63 (m, 2 H, H-6'e, 5f), 3.57 (t, 1 H, $J_{2,3}$=8.4 Hz, H-3e), 3.45–3.40 (m, 3 H, H-2e, 4e, 5e); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 165.7, 165.2, 138.6, 138.5, 138.1, 137.5, 137.0, 133.3, 133.2, 129.9, 129.8, 129.5, 129.3, 129.1, 128.5, 128.4, 128.3, 128.1, 128.0, 127.9, 127.8, 127.7, 127.6, 126.3, 102.5, 101.6, 101.4, 84.7, 82.2, 78.8, 77.8, 75.7, 75.0, 74.9, 74.7, 72.7, 72.3, 71.1, 68.8, 68.4, 67.2, 66.6, 29.8; MALDI MS: *m/z*: calcd for $C_{61}H_{58}O_{13}Na$: 1,021.38; found: 1,021.49 [*M* + Na] $^+$.

Benzyl 2,3-di-O-benzyl-4,6-O-benzylidene-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**20**) To a solution of compound **18** (1.25 g, 1.25 mmol) in mixed solvent (MeOH/THF=15:7.5 ml) was added sodium methoxide (28% in MeOH; 24 mg). The mixture was stirred for 7.5 h at ambient temperature, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH= 50:1). The reaction mixture was neutralized with Dowex (H^+) and filtered through cotton. The combined filtrate and washings was concentrated under diminished pressure. To a solution of the residue in DMF (12.5 ml) were added sodium hydride 60% (200 mg, 5.00 mmol) and benzyl bromide (594 μl, 5.00 mmol). The mixture was stirred for 3 h at ambient temperature, as the proceeding of the reaction was monitored by TLC (toluene/EtOAc= 12:1). Triethylamine and ammonium chloride were added to the reaction mixture. The reaction mixture was washed with H_2O and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel (toluene/EtOAc=40:1) to give **20** (1.07 g, 88%).; $[\alpha]_D$=−21.7° (*c* 1.1, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.49–7.21 (m, 35 H, 7 Ph), 5.56 (s, 1 H, >C*H*Ph), 4.96–4.69 (m, 10 H, 10 CH*H*Ph), 4.59 (d, 1 H, $J_{1,2}$=8.2 Hz, H-1f), 4.54–4.45 (m, 3 H, H-1e, 2 CH*H*Ph), 4.33 (dd, 1 H, J_{gem}=9.3 Hz, $J_{5,6}$=4.8 Hz, H-6f), 4.16 (d, 1 H, J_{gem}=11.0 Hz, H-6e), 3.79–3.72 (m, 2 H, H-6'e, 6'f), 3.68–3.64 (m, 3 H, H-2f, 4f, 5f), 3.57 (t, 1 H, $J_{2,3}$=8.5 Hz, $J_{3,4}$=9.0 Hz, H-3e), 3.50–3.47 (m, 2 H, H-2e, 2f), 3.44 (t,

1 H, $J_{3,4}$=9.6 Hz, $J_{4,5}$=9.6 Hz, H-4e), 3.35 (m, 1 H, H-5e); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 138.6, 138.6, 138.8, 138.1, 137.6, 137.5, 129.1, 128.7, 128.5, 128.4, 128.3, 128.3, 128.3, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.8, 127.7, 127.6, 126.1, 104.3, 102.7, 101.3, 84.8, 82.4, 82.1, 81.6, 81.0, 78.3, 77.3, 75.8, 75.4, 75.2, 75.1, 74.9, 71.3, 68.9, 66.1, 29.8; MALDI MS: *m/z*: calcd for $C_{61}H_{62}O_{11}Na$: 993.42; found: 993.50 [M + Na] $^+$.

Benzyl 2,3,6-tri-O-benzyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**21**) To a solution of compound **20** (82 mg, 84.5 μmol) in CH_2Cl_2 (845 μl) were added triethylsilane (162 μl, 1.01 mmol) and boron trifluoride diethyl etherate (BF_3·OEt_2; 21.4 μl, 169 μmol). The mixture was stirred for 1.5 h at ambient temperature, as the proceeding of the reaction was monitored by TLC (toluene/EtOAc=12:1). The reaction mixture was diluted with $CHCl_3$ and washed with sat. $NaHCO_3$, H_2O and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel (toluene/EtOAc=20:1) to give **21** (70 mg, 85%).; $[\alpha]_D$=−12.9° (*c* 1.0, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 7.35–7.21 (m, 35 H, 7 Ph), 5.01–4.69 (m, 10 H, 10 CH*H*Ph), 4.59–4.51 (m, 3 H, H-1f, 2 CH*H*Ph), 4.46 (d, 1 H, $J_{1,2}$=9.6 Hz, H-1e), 4.19 (d, 1 H, J_{gem}=11.0 Hz, H-6e), 3.74–3.58 (m, 6 H, H-6′e, 3f, 4f, 5f, 6f, 6′f), 3.50–3.39 (m, 5 H, H-2f, 2e, 3e, 4e, 5e), 2.54 (s, 1 H, -OH); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 138.9, 138.7, 138.5, 138.5, 138.2, 138.0, 137.6, 128.6, 128.5, 128.5, 128.4, 128.3, 128.2, 128.1, 128.0, 128.0, 127.9, 127.8, 127.7, 104.1, 102.7, 84.8, 84.2, 82.4, 81.6, 78.4, 77.3, 75.8, 75.4, 75.3, 75.1, 74.9, 74.8, 74.1, 73.8, 71.8, 71.3, 68.8, 29.8; MALDI MS: *m/z*: calcd for $C_{61}H_{64}O_{11}Na$: 995.43; found: 995.38 [M + Na] $^+$.

Benzyl {methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-4-O-acetyl-2,6-di-O-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**22**) To a solution of compound **13** (107 mg, 102 μmol) and **21** (200 mg, 206 μmol) in CH_2Cl_2 (5.0 ml) was added molecular sieves 4 Å (1.00 g). The suspension was stirred for 1 h and cooled to 0°C. To the mixture was added trimethylsilyl trifluoromethanesulfonate (TMSOTf; 3.7 μl, 20 μmol) and stirring was continued for 1 h. Completion of the reaction was confirmed by TLC ($CHCl_3$/MeOH=20:1). The reaction mixture was filtered through Celite. The combined filtrate and washings was extracted with $CHCl_3$, and the organic layer was washed with sat. Na_2CO_3 and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH=75:1) to give **22** (170 mg, 86%).; $[\alpha]_D$=+ 2.0° (*c* 0.5, $CHCl_3$); ^{1}H-NMR (500 MHz, $CDCl_3$): δ 8.24–7.15 (m, 45 H, 9 Ph), 5.66 (m, 1 H, H-8b), 5.31 (t, 1 H, $J_{1,2}$=8.0 Hz, $J_{2,3}$= 9.7 Hz, H-2a), 5.18 (dd, 1 H, H-7b), 5.13 (d, 1 H, $J_{1,2}$=8.0 Hz, H-1a), 5.06 (d, 1 H, $J_{3,4}$=3.4 Hz, H-4a), 4.99 (d, 1 H, CH*H*Ph), 4.92–4.66 (m, 12 H, H-3a, 4b, NH, 9 CH*H*Ph), 4.49–4.36 (m, 7 H, H-6'a, 1e, 1f, 4 CH*H*Ph), 4.29 (d, 1 H, H-9'b), 4.13–3.88 (m, 6 H, H-5a, 6a, 5b, 9b, H-6′ of Glc units), 3.77 (q, 1 H, H-5b), 3.71 (s, 1 H, OMe), 3.67–3.35 (m, 10 H, H-6b, Glc units), 3.22 (m, 1 H, H-5 of Glc units), 2.52 (dd, 1 H, J_{gem}=12.6 Hz, $J_{3eq,4}$=4.6 Hz H-3b$_{eq}$), 2.13–1.43 (m, 19 H, 6 Ac, H-3b$_{ax}$) ^{13}C-NMR (125 MHz, $CDCl_3$) δ 170.8, 170.7, 170.3, 170.2, 170.1, 168.0, 165.4, 165.1, 139.1, 138.6, 138.4, 138.0, 137.6, 133.3, 133.0, 130.3, 130.0, 129.8, 129.7, 128.6, 128.4, 128.3, 128.3, 128.2, 128.1, 128.1, 127.9, 127.9, 127.7, 127.6, 127.4, 127.3, 127.2, 127.1, 103.7, 102.7, 100.4, 96.9, 84.7, 82.9, 82.3, 81.6, 78.2, 76.3, 75.7, 75.2, 75.1, 74.9, 74.8, 74.8, 74.4, 72.8, 71.7, 71.5, 71.2, 70.4, 69.4, 69.0, 68.5, 67.4, 67.0, 66.5, 62.5, 61.2, 53.0, 48.8, 37.3, 29.7, 23.2, 21.3, 20.8, 20.7, 20.7, 20.3; MALDI MS: *m/z*: calcd for $C_{103}H_{111}O_{31}NNa$: 1,880.70; found: 1,880.96 [M + Na] $^+$.

Benzyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-galactopyranosyl-(1→4)-{methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-2,6-di-O-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**23**) To a solution of compound **14** (58 mg, 43 μmol) and **21** (84 mg, 86 μmol) in CH_2Cl_2 (2.0 ml) was added molecular sieves 4 Å (165 mg). The suspension was stirred for 1 h at ambient temperature and cooled to 0°C. To the mixture was added TMSOTf (1.6 μL, 8.6 μmol) and stirring was continued for 3.5 h. Completion of the reaction was confirmed by TLC ($CHCl_3$/MeOH=15:1). Triethylamine was then added to quench the reaction. The reaction mixture was filtered through Celite. The combined filtrate and washings was extracted with $CHCl_3$, and the organic layer was washed with sat. $NaHCO_3$ and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH=50:1) to give **23** (70 mg, 76%).; $[\alpha]_D$=−11.0° (*c* 0.76, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.01–7.15 (m, 45 H, 9 Ph), 5.97 (d, 1 H, NH-c), 5.49 (dd, 1 H, $J_{3,4}$=2.7 Hz, H-3c), 5.40 (m, 1 H, H-8b), 5.37 (d, 1 H, $J_{3,4}$=2.7 Hz, H-4c), 5.34 (t, 1 H, $J_{1,2}$= 10.2 Hz, H-2a), 5.25 (d, 1 H, H-7b), 5.14 (br d, 1 H, NH-b), 5.06 (d, 1 H, $J_{1,2}$=8.9 Hz, H-1c), 5.00 (dt, 1 H, $J_{3eq,4}$= 4.8 Hz, H-4b), 4.95–4.88 (m, 4 H, 4 CH*H*Ph), 4.82–4.80 (m, 3 H, 3 CH*H*Ph), 4.79 (d, 1 H, $J_{1,2}$=10.2 Hz, H-1a), 4.72 (t, 2 H, 2 CH*H*Ph), 4.67 (d, 1 H, CH*H*Ph), 4.62 (q, 1 H, H-6c), 4.51 (d, 1 H, CH*H*Ph), 4.47 (d, 1 H, CH*H*Ph), 4.43 (d, 1 H, CH*H*Ph), 4.40 (d, 1 H, $J_{1,2}$=8.2 Hz, H-1f), 4.37 (d, 1 H, $J_{1,2}$= 8.2 Hz, H-1e), 4.28 (d, 1 H, CH*H*Ph), 4.19 (t, 1 H, H-5a), 4.15–3.96 (m, 10 H, H-3a, 4a, 6a, 6'a, 5b, 9b, 9'b, 2c, 6'c, 5e), 3.95 (t, 1 H, H-4f), 3.83–3.81 (m, 4 H, OMe, H-6b), 3.64

(t, 1 H, H-5c), 3.63–3.59 (m, 2 H, H-3e, 6e), 3.53 (t, 1 H, H-6'e), 3.50–3.49 (m, 2 H, H-6f, 6'f), 3.47 (t, 1 H, H-3f), 3.44 (t, 1 H, H-4e), 3.39 (t, 1 H, H-2f), 3.36 (t, 1 H, H-2e), 3.14 (m, 1 H, H-5f), 2.22 (dd, 1 H, J_{gem}=13.7 Hz, $J_{3eq,4}$=4.8 Hz, H-3b$_{eq}$), 1.93 (t, 1 H, H-3b$_{ax}$), 2.19–1.75 (9 s, 27 H, 9 Ac); ^{13}C-NMR (100 MHz, $CDCl_3$) δ 170.7, 170.4, 170.3, 170.2, 169.7, 169.6, 168.0, 165.8, 164.1, 138.8, 138.6, 138.5, 138.4, 138.3, 137.9, 137.5, 133.2, 133.1, 129.9, 129.4, 128.4, 128.3, 128.3, 128.2, 128.2, 128.2, 128.1, 128.1, 128.0, 127.8, 127.8, 127.6, 127.5, 127.5, 127.3, 127.0, 103.7, 102.6, 101.1, 100.0, 98.6, 84.6, 82.6, 82.2, 81.6, 78.2, 77.1, 76.2, 76.2, 75.6, 75.1, 74.8, 74.7, 74.3, 73.9, 73.1, 72.0, 72.0, 71.2, 71.1, 70.3, 70.0, 68.9, 68.3, 68.2, 67.2, 67.1, 66.3, 63.3, 62.1, 61.4, 53.1, 51.5, 49.1, 35.8, 29.6, 23.2, 23.1, 21.0, 20.8, 20.7, 20.7, 20.5, 20.4, 20.3; MALDI MS: *m/z*: calcd for $C_{115}H_{128}N_2O_{38}Na$: 2,167.80; found: 2,167.91 [*M* + Na] $^+$.

Benzyl 2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl-(1→3)-2-acetamido-4,6-di-O-acetyl-2-deoxy-β-D-galactopyranosyl-(1→4)-{methyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonate-(2→3)}-2,6-di-O-benzoyl-β-D-galactopyranosyl-(1→4)-2,3,6-tri-O-benzyl-β-D-glucopyranosyl-(1→6)-2,3,4-tri-O-benzyl-β-D-glucopyranoside (**24**) To a solution of compound **15** (105 mg, 64.5 μmol) and **21** (137 mg, 129 μmol) in CH_2Cl_2 (1.9 ml) was added molecular sieves 4 Å (300 mg). The suspension was stirred for 30 min and cooled to 0°C. To the mixture was added TMSOTf (1.2 μl, 6.5 μmol) and stirring was continued for 45 min. Completion of the reaction was confirmed by TLC (toluene/EtOAc=7:1). The reaction mixture was filtered through Celite. The combined filtrate and washings was extracted with $CHCl_3$, and the organic layer was washed with sat. Na_2CO_3 and brine, dried over Na_2SO_4 and concentrated. The residue was purified with column chromatography on silica gel ($CHCl_3$/MeOH= 200:3) to give **24** (110 mg, 69%).; $[\alpha]_D$=+ 0.0° (*c* 0.8, $CHCl_3$); ^{1}H-NMR (600 MHz, $CDCl_3$): δ 8.18–7.09 (m, 45 H, 9 Ph), 5.88 (d, 1 H, $J_{5,NH}$=6.3 Hz, NH-c), 5.66 (m, 1 H, H-8b), 5.38–5.31 (m, 3 H, H-2a, 4c, 4d), 5.19 (dd, 1 H, $J_{6,7}$= 2.3 Hz, $J_{7,8}$=9.7 Hz, H-7b), 5.15 (d, 1 H, $J_{1,2}$=8.0 Hz, H-1c), 5.11–5.07 (m, 2 H, H-3a, 2d), 5.01–4.58 (m, 17 H, H-6a, 4b, 3c, 1d, 3d, 1f, NH-b, 10 CH*H*Ph), 4.48–4.37 (m, 5 H, $J_{1,2}$=7.4 Hz, H-1a, $J_{1,2}$=8.1 Hz, H-1e, 6e, 2 CH*H*Ph), 4.29–4.22 (m, 3 H, H-9b, 2 CH*H*Ph), 4.12–3.25 (m, 28 H, H-4a, 5a, 6a, 6'a, 5b, 6b, 9'b, 2c, 5c, 6c, 6'c, 5d, 6d, 6'd, 2e, 3e, 4e, 5e, 6'e, 2f, 3f, 4f, 5f, 6f, 6'f, -OMe), 2.73 (dd, 1 H, J_{gem}= 12.6 Hz, $J_{3eq,4}$=4.3 Hz, H-3b$_{eq}$), 2.19–1.49 (m, 37 H, H-3b$_{ax}$, 12 Ac); ^{13}C-NMR (150 MHz, $CDCl_3$) δ 172.1, 170.9, 170.7, 170.5, 170.3, 170.2, 170.2, 170.0, 169.3, 168.4, 165.5, 165.1, 138.9, 138.6, 138.6, 138.5, 138.1, 137.6, 133.4, 133.1, 130.3, 130.1, 130.0, 129.6, 128.7, 128.4, 128.2, 128.0, 128.0, 127.9, 127.8, 127.7, 127.7, 127.6, 127.3, 127.3, 103.8, 102.7, 101.1, 100.7, 99.0, 97.9, 84.7, 83.2, 82.4, 81.8, 78.3, 75.7, 75.2, 75.0, 75.0, 74.8, 74.5, 73.9, 73.6, 72.9, 72.2, 71.9, 71.6, 71.3, 71.0, 70.6, 69.2, 69.1, 69.0, 68.6, 67.1, 66.9, 66.6, 63.2, 62.8, 62.6, 61.0, 55.3, 52.8, 49.3, 36.9, 29.8, 24.0, 23.2, 22.8, 21.4, 20.9, 20.8, 20.8, 20.8, 20.7, 20.7, 20.4, 20.3, MALDI MS: *m/z*: calcd for $C_{127}H_{144}N_2O_{46}Na$: 2,455.89; found: 2,455.52 [*M* + Na] $^+$.

β-D-Galactopyranosyl-(1→3)-2-acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-{5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid-(2→3)}-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-β-D-glucopyranose (**1**) To a solution of compound **24** (95 mg, 39 μmol) in MeOH (1.6 ml) was added sodium methoxide (28% in MeOH; 14 mg). The mixture was stirred for 74 h under reflux condition, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH/H_2O=3:2:0.3). H_2O (1.6 ml) was then added and stirring was continued for 14 h at ambient temperature. The reaction mixture was neutralized with Dowex (H^+) and filtered through cotton. The combined filtrate and washings was concentrated under diminished pressure to give a syrup compound. To a solution of the residue in H_2O (1.4 ml) was added palladium hydroxide [$Pd(OH)_2$] (20 wt% Pd on carbon; 345 mg). The mixture was vigorously stirred for 4 h at 40°C under hydrogen atmosphere, as the proceeding of the reaction was monitored by TLC (1-BuOH/MeOH/H_2O= 2:1:1). The reaction mixture was filtered through Celite, and the combined filtrate and washings was concentrated. The residue was purified with gel filtration column chromatography (Sephadex LH-20, H_2O as eluent) to give **1** (43 mg, 99%).; $[\alpha]_D$=+ 0.1° (*c* 1.0, H_2O); ^{1}H-NMR (600 MHz, CD_3OD): δ 5.16 (d, 1 H, $J_{1,2}$=3.7 Hz, H-1e), 4.79 (d, 1 H, H-1c), 4.57 (d, 1 H, $J_{1,2}$=8.0 Hz, H-1d), 4.49–4.45 (m, 3 H,H-1a, 1b, 1f), 4.15–3.19 (m, 39 H, ring H), 2.62 (dd, 1 H, H-3b$_{eq}$), 1.99 and 1.96 (2 s, 6 H, 2 Ac), 1.87 (m, 1 H, H-3b$_{ax}$), ^{13}C-NMR (150 MHz, CD_3OD) δ 175.0, 174.8, 174.1, 106.1, 105.7, 105.5, 104.1, 104.0, 103.4, 101.8, 97.0, 95.3, 94.2, 93.0, 91.5, 84.4, 81.2, 78.1, 77.6, 76.5, 75.1, 74.8, 74.5, 74.3, 73.9, 73.5, 73.4, 72.6, 72.1, 71.5, 70.8, 70.2, 68.9, 68.0, 67.1, 61.2, 61.0, 60.7, 59.7, 59.3, 58.8, 52.5, 51.6, 48.8, 47.5, 28.7, 25.9, 23.5; MALDI MS: *m/z*: calcd for $C_{43}H_{72}N_2O_{34}$: 1160.40; found: 1159.75 [*M*-H]$^-$.

2-Acetamido-2-deoxy-β-D-galactopyranosyl-(1→4)-{5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid-(2→3)}-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-D-glucopyranose (**2**) To a solution of compound **23** (38 mg, 18 μmol) in MeOH (2.0 ml) was added catalytic amounts of sodium methoxide (10 mg). The mixture was stirred for 96 h under reflux conditions, as

the proceeding of the reaction was monitored by TLC (1-BuOH/MeOH/H_2O=4:1:1). H_2O was then added and stirring was continued for 10 h at ambient temperature. The reaction mixture was neutralized with Dowex (H^+) and filtered through cotton. The combined filtrate and washings was concentrated under diminished pressure to give a syrupy compound. The residue was purified by gel filtration column chromatography on Sephadex LH-20 (MeOH) to give a white solid. To a solution of the solid in MeOH/H_2O (2.5/1 ml) was added palladium hydroxide [$Pd(OH)_2$] (20 wt% Pd on carbon; 40 mg). The mixture was vigorously stirred overnight at 40°C under hydrogen atmosphere, as the proceeding of the reaction was monitored by TLC (1-BuOH/MeOH/H_2O=2:1:1). The reaction mixture was filtered through Celite. The combined filtrate and washings was concentrated. The residue was purified with gel filtration column chromatography (Sephadex LH-20, MeOH/H_2O= 1:1 as eluent) using MeOH as eluent, to give **2** (18 mg, 98%).; $[\alpha]_D$=+19.4° (*c* 1.7, MeOH:H_2O=1:1); ^{1}H-NMR (500 MHz, CD_3OD/D_2O=1:1): δ 2.69 (dd, 1 H, J_{gem}=11.4 Hz, $J_{3eq,4}$=4.6 Hz, H-3b_{eq}), 2.04 and 2.02 (2 s, 6 H, 2 NAc), 1.91 (t, 1 H, H-3b_{ax}); ^{13}C-NMR (125 MHz, CD_3OD/D_2O= 1:1) δ 176.0, 175.4, 175.0, 103.9, 103.9, 103.7, 102.9, 97.3, 93.4, 80.0, 78.5, 77.0, 76.1, 75.8, 75.6, 75.4, 75.1, 71.0, 70.8, 69.8, 69.6, 69.5, 69.1, 64.2, 62.3, 61.5, 61.2, 53.5, 53.0, 49.5, 49.4, 48.4, 38.0, 23.6, 22.8; MALDI MS: *m/z*: calcd for $C_{37}H_{61}N_2O_{29}$: 997.33; found: 997.25 [*M*-H]$^-$.

{5-Acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosylonic acid-(2→3)}-β-D-galactopyranosyl-(1→4)-β-D-glucopyranosyl-(1→6)-D-glucopyranose (**3**) To a solution of compound **22** (45 mg, 24 μmol) in MeOH (3.0 ml) was added sodium methoxide (28% in MeOH; 11 mg). The mixture was stirred for 48 h at 45°C, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH=5:1). H_2O (1.0 ml) was then added and stirring was continued for 18 h at 45°C. The reaction mixture was neutralized with Dowex (H^+) and filtered through cotton. The combined filtrate and washings was concentrated under diminished pressure to give a syrupy compound. To a solution of the residue in H_2O (2.0 ml) was added palladium hydroxide [$Pd(OH)_2$] (20 wt% Pd on carbon; 100 mg). The mixture was stirred for 8 h at ambient temperature under hydrogen atmosphere, as the proceeding of the reaction was monitored by TLC ($CHCl_3$/MeOH/H_2O=3:1:0.1). The reaction mixture was filtered through Celite. The combined filtrate and washings was concentrated. The residue was purified with gel filtration column chromatography (Sephadex LH-20, H_2O as eluent) to give **3** (14 mg, 76%).; $[\alpha]_D$=+ 8.3° (*c* 0.6, H_2O); ^{1}H-NMR (400 MHz, D_2O): δ 5.21 (d, 1 H, $J_{1,2}$=3.7 Hz, H-1e), 4.64–4.51 (m, 2 H, H-1a, 1f), 4.21–2.87 (m, 25 H, ring H), 2.75 (dd, 1 H, J_{gem}=12.0 Hz, $J_{3eq,4}$=4.6 Hz, H-3b_{eq}), 2.02 (s, 3 H, Ac), 1.77 (m, 1 H, H-3b_{ax}), ^{13}C-NMR (100 MHz, D_2O) δ 177.7, 176.6, 105.4, 105.2, 102.5, 98.7, 94.8, 80.9, 78.4, 77.9, 77.6, 77.5, 77.5, 77.0, 76.7, 75.6, 75.5, 75.4, 74.5, 74.1, 73.1, 72.2, 72.1, 71.5, 71.4, 71.1, 70.8, 70.2, 65.3, 65.2, 63.7, 62.7, 57.1, 54.4, 42.4, 24.8, 21.8, 17.7; MALDI MS: *m/z*: calcd for $C_{29}H_{24}NO_{24}$: 795.26; found: 794.24 [*M*-H]$^-$.

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Research Report

Distributions of glucuronyltransferases, GlcAT-P and GlcAT-S, and their target substrate, the HNK-1 carbohydrate epitope in the adult mouse brain with or without a targeted deletion of the GlcAT-P gene

Mitsuhiro Inoue[a], *Keiko Kato*[b,c,*], *Hitomi Matsuhashi*[c], *Yasuhiko Kizuka*[a], *Toshisuke Kawasaki*[a], *Shogo Oka*[a]

[a]*Department of Biological Chemistry, Graduate School of Pharmaceutical Sciences, Kyoto University, Kyoto, 606-8501, Japan*
[b]*Department of Structural and Functional Biosciences for Animals, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka, 599-8531, Japan*
[c]*Division of Structural Cell Biology, Nara Institute of Science and Technology (NAIST), 8916-5 Takayama, Ikoma, Nara, 630-0101, Japan*

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ABSTRACT

The HNK-1 carbohydrate epitope, a sulfated glucuronic acid at the non-reducing terminus of glycans, is expressed on glycoproteins and glycolipids and modulates neurite outgrowth and synaptic plasticity by affecting the adhesive and anti-adhesive properties. It is known that the HNK-1 carbohydrate is synthesized through two key enzymes, glucuronyltransferases (GlcAT-P and GlcAT-S). In the present study, we investigated the localization of GlcAT transcripts and HNK-1 carbohydrate in the adult mouse brain with or without GlcAT-P gene using *in situ* hybridization histochemistry and immunohistochemistry. Region-specific expression patterns of both GlcAT transcripts were observed. Strong expression of GlcAT-P and moderate expression of GlcAT-S were seen in neuronal cells of several nuclei of limbic-related regions and of the sensory system and the cerebellum. It was shown histologically that the localization of HNK-1 carbohydrate paralleled the pattern of expression of GlcAT

* *Corresponding author.* Present address: Department of Structural and Functional Biosciences for Animals, Graduate School of Life and Environmental Sciences, Osaka Prefecture University, 1-1 Gakuen-cho, Sakai, Osaka, 599-8531, Japan. Fax: +81 72254 9496.
E-mail address: kato@vet.osakafu-u.ac.jp (K. Kato).

Abbreviations: GlcA, glucuronic acid; GlcAT-P, glucuronyltransferases P (AB055781); GlcAT-S, glucuronyltransferase S (AB055902); HNK-1, $HSO_3$3GlcAβ1-3Galβ1-4GlcNAc; NCAM, neural cell adhesion molecule; CNS, central nervous system; 3n, oculomoter nu.; aca, anterior commissure; AD, anterodorsal thalamic nu.; Arc, arcuate hypothalamic nu.; AO, anterior olfactory nu.; BST, bed nu. of stria terminallis; CA1–3, subfield CA1–3 of Ammon's horn; Cbn, cerebellar nu.; cc, corpus callosum; Cg, cingulate cortex; Cu, cuneate nu.; DB, diagonal band; DC, dorsal cochlear nu.; DG, dentate gyrus.; DM, dorsomedial hypothalamic nu.; Ect, ectorhinal cortex; En, endopiriform cortex; fr, fasciculus retroflexus; Gl, glomerular layer of olfactory bulb; glc, granular cell layer of the dentate gyrus; Gr, gracile nu.; Hip, hippocampus; IC, inferior colliculus; IO, inferior olive; IP, interpeduncular nu.; LL, lateral lemniscus; LPB, lateral parabrachial nu.; LRt, lateral reticular nu.; LS, lateral septal nu.; MD, mediodorsal thalamic nu.; MG, medial geniculate nu.; MHb, medial habenular nu.; Mi, mitral cell layer of olfactory bulb; ml, molecular layer of the dentate gyrus; MPO, medial preoptic nu.; O, orbital cortex; PAG, periaquiductal gray; PB, parabrachial nu.; PBG, parabigeminal nu.; pcl, pyramidal cell layer of the hippocampus; Pir, piriform cortex; Pn, pontine nuclei; PoDG, polymorphic layer of the dentate gyrus; Pr5, principal sensory trigeminal nu.; PVA, anterior part of paraventricular thalamic nu.; Rt, reticular nucleus of the thalamus; RtTg, reticulotegmental nu. of pons; S, subiculum; SC, superior colliculus; SFO, subfornical organ; s-l, stratum lucidum; sm, stria medullaris of thalamus; s-o, stratum oriens; SO, superior olivary nu.; Sol, solitary tract nu.; Sp5, spinal trigeminal nu.; s-r, stratum radiatum; Tha, thalamus; VC, ventral cochlear nu.; VMH, ventromedial hypothalamic nu

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transcripts in the brain. Additionally, the localization of HNK-1 carbohydrate was restricted partially in the brain of GlcAT-P-deficient mice, while the HNK-1 carbohydrate was widely distributed over most of the brain of wild-type mice. The present study provides a new framework for understanding the network constructed by the HNK-1 carbohydrate in the central nervous system.

1. Introduction

The central nervous system performs the most complex and dynamic biological functions. These functions are produced by complex neural connections resulting from the formation and maintenance of the vast array of synapses and cell–cell interactions, which depend on specific interactions between the extracellular matrix and cell membrane components. Carbohydrates on glycoproteins and glycolipids play important roles in a number of these interactions by enhancing either the adhesive or anti-adhesive properties of molecules involved in cellular adhesion (Pizzorusso et al., 2002; Rhodes and Fawcett, 2004). The HNK-1 carbohydrate epitope, a sulfated trisaccharide, $HSO_3$3GlcAβ1-3Galβ1-4GlcNAc (Chou et al., 1986; Voshol et al., 1996) is found on a number of glycoproteins, including neural cell adhesion molecule (NCAM) (Ong et al., 2002), L1, myelin-associated glycoprotein (Kruse et al., 1984), tenascin-C, and tenascin-R (Kruse et al., 1985), tissue plasminogen activator (Zamze et al., 2001), and glycolipids (Chou et al., 1986). Cell-biologically, the HNK-1 carbohydrate is thought to function in the modulation of neurite outgrowth (Martini et al., 1992), adhesion between neurons and glial cells (Kunemund et al., 1988), and synaptic plasticity (Dityatev and Schachner, 2003; Yamamoto et al., 2002). On the other hand, there have been few investigations concerning the HNK-1 carbohydrate in the adult brain *in vivo* except for ones focused on the hippocampus. One report showed that the immunoreactivity of the anit-HNK-1 carbohydrate antibody is detected as diffuse staining in the neuropil and individual somata in almost the whole brain (Yamamoto et al., 1988), but there is little knowledge about critical sites where the HNK-1 carbohydrate is expressed and to which it is conveyed in the brain network. The present study revealed critical regions expressing the HNK-1 carbohydrate based on histological observations and revealed the distributions of mRNAs of enzymes catalyzing HNK-1 synthesis. Two glucuronyltransferases, GlcAT-P and GlcAT-S, are key enzymes in the biosynthesis of HNK-1 carbohydrate, and catalyze the transfer of glucuronic acid (GlcA) from UDP-GlcA to Galβ1-4GlcNAc (Seiki et al., 1999; Shimoda et al., 1999; Terayama et al., 1997, 1998). Recently, we characterized the acceptor specificities of the two glucuronyltransferases using various oligosaccharides, suggesting the possibility that the two glucuronyltransferases synthesize structurally different HNK-1 carbohydrates (Kakuda et al., 2005). While it has been reported that GlcAT-P catalyzes HNK-1 synthesis mainly in the brain (Terayama et al., 1998), there have been no investigations of the specific distributions of each GlcAT transcript, and it has not been possible to discriminate between the HNK-1 carbohydrate produced by GlcAT-P and that produced by GlcAT-S. Recently, we generated GlcAT-P-deficient mice (Yamamoto et al., 2002), which permit us to determine the region-specificities of HNK-1 carbohydrate based on the expression of either GlcAT transcript in the brain. In the present study, we investigated the formation of the HNK-1 carbohydrate network in the central nervous system (CNS).

2. Results

2.1. *Distribution of GlcAT transcripts and HNK-1 in the adult mouse brain*

In normal adult mouse brain, most of the cells labeled with GlcAT-P and GlcAT-S cRNAs were ones showing representative neuronal shapes among cells stained with thionine. Table 1 shows scores based on a comparison of the relative signal intensities not only among different brain regions but also between GlcAT-P and GlcAT-S riboprobes, and Fig. 1 shows representative sections. There was no discrepancy concerning the levels of the transcripts between the present *in situ* hybridization and the previous Northern blot analyses (Terayama et al., 1997; Yamamoto et al., 2002). GlcAT-P mRNA was expressed widely in the mouse brain, while the expression of GlcAT-S mRNA was restricted. Regarding the distribution of GlcAT-P mRNA, especially, very strong labeling of neurons was detected in several nuclei of limbic-related regions, several sensory systems, and the cerebellum. Among the limbic-related regions, the anterior olfactory nucleus (AO; Fig. 1A-b), the piriform cortex (Pir; Fig. 1A-c), the lateral septum (LS; Figs. 1A-c and i), the hippocampus (Fig. 1A-d), the habenular nucleus (MHb; Fig. 1A-d), and the interpeduncular nucleus (IP; Fig. 1A-e) expressed GlcAT-P mRNA strongly. Among the sensory systems, there were the intense signals in the cochlear nucleus (VC and DC; Figs. 1A-f and g), the lateral lemuniscus (LL; Fig. 1A-j), and the inferior colliculus (IC; Fig. 1A-f) in the auditory system, the parabigeminal nucleus (PBG; Fig. 1A-j) in the visual system, the parabrachial nuclei (LPB; Fig. 1A-f) that function as gustatory relays, and the trigeminal sensory system (Pr5 and Sp5; Figs. 1A-f, g, and h). Concerning the expression of GlcAT-S mRNA, the transcript was detected in restricted areas among regions showing the expression of GlcAT-P mRNA and especially, the CA2/CA3-subfields (CA3; Fig. 1B-o), the ectorhinal cortex (Ect; Fig. 1B-o), and several nuclei of the thalamus (MD; Fig. 1B-n). In addition, the subfornical organ (SFO; Fig. 1B-n), the arcuate hypothalamic nucleus (Arc; Fig. 1B-o), and the medial geniculate of the thalamus (MG; Fig. 1B-p) expressed GlcAT-S mRNA very strongly, but expressed little GlcAT-P mRNA. We also investigated the distributions of GlcAT-P and GlcAT-S transcripts in the GlcAT-P-deficient mouse brain. There was, as expected, no signal with GlcAT-P cRNA probe in the GlcAT-P-deficient mouse brain. There was no difference in regional distributions