Bioorganic & Medicinal Chemistry 18 (2010) 3760–3766

Contents lists available at ScienceDirect

Bioorganic & Medicinal Chemistry

journal homepage: www.elsevier.com/locate/bmc

Probe design and synthesis of Galβ(1→3)[NeuAcα(2→6)]GlcNAcβ(1→2)Man motif of *N*-glycan

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ARTICLE INFO

Article history:
Received 1 April 2010
Revised 20 April 2010
Accepted 21 April 2010
Available online 28 April 2010

Keywords:
Oligosaccharide synthesis
N-Glycan
Sialic acid
Glycosylation
Glycodendrimer
Glycocluster
Click reaction

ABSTRACT

Synthesis and clusterization of Galβ(1→3)[NeuAcα(2→6)]GlcNAcβ(1→2)Man motif of the *N*-glycan, as the molecular probes for their biological evaluation, are reported. Key step is the quantitative and the completely α-selective sialylation of the C5-azide *N*-phenyltrifluoroacetimidate with the disaccharide acceptor, Galβ(1→3)GlcNTroc. Clusterization of the 16 molecules of trisaccharide motif was also achieved by the 'self-activating click reaction'. These probes could efficiently be labeled by biotin and/or other fluorescence- or radioactive reporter groups through either cross metathesis, acylation, Cu(I)-mediated Huisgen [2+3]-cycloaddition, or the azaelectrocyclization to utilize the various biological techniques.

1. Introduction

Among the various types of oligosaccharide structures, asparagine-linked oligosaccharides (*N*-glycans) are prominent in terms of diversity and complexity. In particular, *N*-glycans containing sialic acid residues are involved in a variety of important physiological events, including cell–cell recognition, adhesion, signal transduction, and quality control.[1] For examples, it has long been known that the sialic acids in *N*-glycans on soluble proteins, which usually contain Neuα(2→6)Gal-linkages, enhance circulatory residence,[2,3] that is, *N*-glycan-engineered erythropoietin (EPO)[2b] or insulin[2c] exhibits a remarkably higher stability in serum, which effects the prolonged bioactivity. Antibody-dependent cellular cytotoxicity (ADCC) and/or complement-dependent cytotoxicity (CDC) has also been proposed to be modulated by the sialic acids of *N*-glycans in immunoglobulin (IgG) through Siglec interactions by glycosylating or glycosidazing the sialic acids.[4] On the other hands, sialic acid linking to the C3-hydroxyl of galactose could usually be found in the nerve systems, for example, the brain gangliosides, which are known to stabilize the myelin and control the nerve generation.[5] The α(2→8)- and α(2→9)-polysialic acids introduced at non-reducing end of the brain *N*-glycans, that is, on the neural cell adhesion molecule (NCAM), are also known to regulate the neural development by 'electronically' and/or 'sterically' inhibiting the neural cell interactions.[6]

Mono-sialylated Galβ(1→3)[NeuAcα(2→6)]GlcNAc and di-sialylated NeuAcα(2→3)Galβ(1→3)[NeuAcα(2→6)]GlcNAc structures were found in human milk oligosaccharides[7] and the *N*-glycan chains of several glycoproteins[8] (Fig. 1). However, physiological role of NeuAcα(2→6)GlcNAc structure or lectins that recognize this motif have not been known yet. Siglec families[9] are the candidates for the recognition of this motif. However, the similar glycan with the structure of Galβ(1→3)[NeuAcα(2→6)]GlcNAcβ-(1→3)Galβ(1→4)Glc showed only weak interaction with either Siglec-7 or Siglec-9.[10]

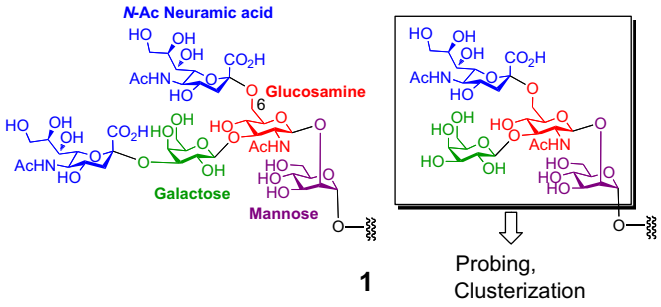

Figure 1. Partial structure of *N*-glycan having NeuAcα(2→6)GlcNAc.

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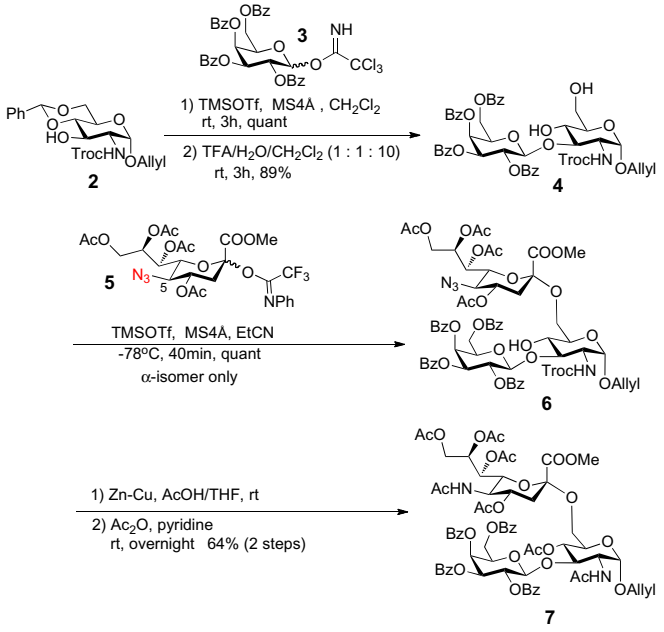
doi:10.1016/j.bmc.2010.04.067

These results motivated us to design and synthesize the tri- and tetrasaccharides in **1** as the molecular probes, providing an attractive opportunity for evaluation of their biological functions, for example, biotin-probes to 'fish-out' the interacting lectins, and/or the protein bioconjugate to prepare the antibodies to visualize the localization of the glycan. In this paper, we will report the efficient synthesis of the tri- and tetrasaccharides in **1** as the molecular probes to be applicable to the various biological investigation. The dendrimer-type cluster, where the 16 molecules of trisaccharide non-reducing end structure was also synthesized in order to enhance the interaction with the target lectins by using the well-known glycan multi-valency effects.[1,11]

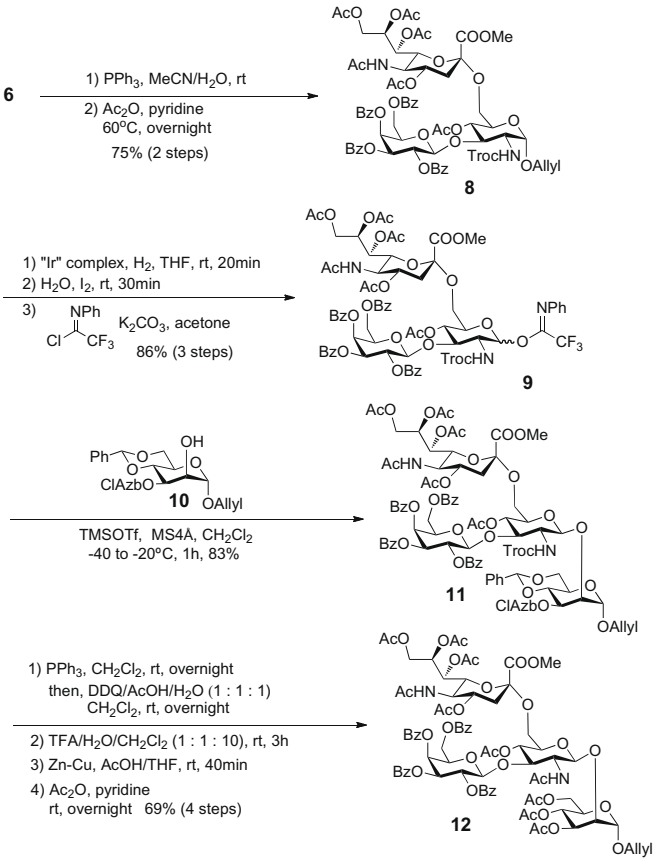
Recently, various studies have been reported for efficient synthesis of *N*-glycan.[12] We have reported the library-directed solid-phase synthesis of sialic acid containing *N*-glycan and microfluidic synthesis of the fragments used for solid-phase synthesis. In the present study, we employed solution-phase approach in order to obtain enough amount of the probes. The key to the synthesis of the trisaccharide and tetrasaccharide motifs in **1** is obviously the α-sialylation of the C6-hydroxyl of glucosamine derivative (Scheme 1). We have developed the efficient and highly stereoselective α(2→6)- and α(2→3)-sialylation of the galactose derivatives by utilizing the sialyl donor having the *N*-phenyl trifluoroacetimide leaving group[13] and the C5-phtalimide or azide functions (such as **5** in Scheme 1) to effect the 'fixed dipole moment effects'.[14,15] Application of microfluidic conditions further makes the α-sialylation to be applicable to α(2→6)- and α(2→3)-sialoside synthesis on 10 g-scale;[15,16] therefore, our sialylation protocol was applied to the disaccharide **4**, Galβ(1→3)GlcNTroc acceptor (Scheme 1). Disaccharide **4** was readily obtained by the glycosylation of perbenzoylated galactose imidate **3** with the *N*-Troc-protected glucosamine acceptor **2** in the presence of TMSOTf as an activator, followed by the hydrolysis of the benzylidene acetal by the treatment with TFA, 89% in two steps. Gratifyingly, when a propionitrile solution of disaccharide **4** and C5-azide imidate **5** was treated with 0.2 equiv of TMSOTf at −78 °C in the conventional flask, the desired trisaccharide **6** was obtained quantitatively as a single α-isomer. The α-configuration of the sialoside linkage[17] was determined based on the $^{3}J_{C1-3Hax}$ of 13.2 Hz [characteristic 3-H signals: δ 2.77 (dd, 1H, *J* = 4.9 Hz, 12.9 Hz, 3-H*eq*^{Neu}) and δ 1.84 (dd, 1H, *J* = 12.9, 12.9 Hz, 3-H*ax*^{Neu}); C-1 signal at δ 167.9]. The current 'batch' sialylation did not necessitate the microfluidic conditions even on the gram-scale synthesis; the trisaccharide **6** could be reproducibly obtained in 90–100%. Presumably, the reduced hydroxyl reactivity of the disaccharide acceptor **4** might not necessitate the precise heat transfer, while the exothermic sialylation with more reactive mono-galactose acceptor[15] caused the severe problems associated with the glycal formation.

Removal of *N*-Troc group and reduction of azide in **6** were simultaneously achieved by the treatment with Zn–Cu in AcOH/THF, and the resulting diamine as well as C4-hydroxyl of the glucosamine were acetylated with Ac_2O in pyridine to give the trisaccharide **7** in 64% for two steps. Trisaccharide **7** is a key compound for the synthesis of the various trisaccharide probes, which were prepared by use of the C=C double bond of the C1-allyl function, via either cross metathesis or the oxidation followed by the acylation of the resulting acid (vide infra).

On the other hand, the tetrasaccharide derivative was synthesized from the trisaccharide **6** by glycosylating with the mannose fragment **10** (Scheme 2). The C5-azide in trisaccharide **6** was selectively converted to the *N*-acetyl group by Staudinger reaction followed by the acetylation of the resulting iminophosphorane (75% for two steps). After C1-allyl group in **8** was removed by the treatment with the activated 'Ir' complex, (1,5-cyclooctadiene) bis(methyldiphenylphosphine)iridium(I)PF_6, followed by the I_2-mediated hydrolysis, the resulting hydroxyl at the reducing end was derivatized to the imidate **9** in 86% (3 steps), by the reaction with *N*-phenyl trifluoroacetimidoyl chloride and K_2CO_3 as a base in wet acetone. Subsequent glycosylation with the C2-hydroxyl of the mannose acceptor **10** in CH_2Cl_2, by using the TMSOTf as an



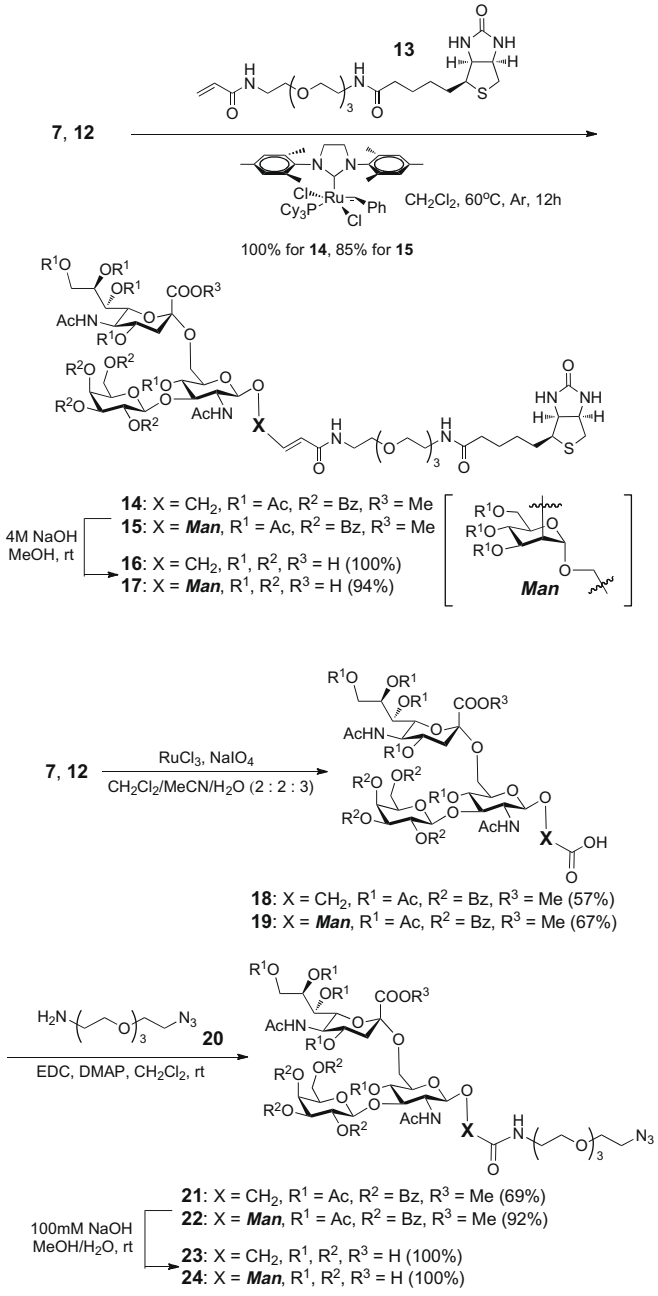
Scheme 1. Synthesis of key intermediate for trisaccharide probes.



Scheme 2. Synthesis of key intermediate for tetrasaccharide probes.

activator, produced the tetrasaccharide **11** in 83%. After the azidochlorobenzyl group at C3-hydroxyl on mannose was removed by the treatment with PPh_3, followed by the DDQ oxidation of the resulting iminophosphorane under the acidic conditions,[18] the straightforward protecting group transformation, namely, hydrolysis of the benzylidene acetal, reductive removal of the *N*-Troc group, and per-acetylation, led to the key tetrasaccharide intermediate **12**, in 69% for four steps.

These tri- and tetrasaccharide intermediates **7** and **12** in hands, the transformations to the biotin-probes were first examined (Scheme 3). Thus, the cross metathesis of the C1-allyl of the trisaccharide **7** with the 5 equiv of acryloyl derivative of biotin **13** in the presence of the 10 mol % Grubbs' 2nd 'Ru' catalyst[19] at 60 °C, successfully provided the trisaccharide/biotin conjugate **14** quantitatively.[20] Removal of all acyl-protecting group and hydrolysis of C1-methoxycarbonyl in **14** were performed quantitatively by the treatment with 4 M NaOH in MeOH, followed by the size-partitioning gel filtration by using Sephadex LH-20. Similarly, the tetrasaccharide **12** could be derivatized to the corresponding biotin probe **17**, 80% for two steps.



Scheme 3. Derivatization to biotin, carboxylic acid- and azide-derivatives.

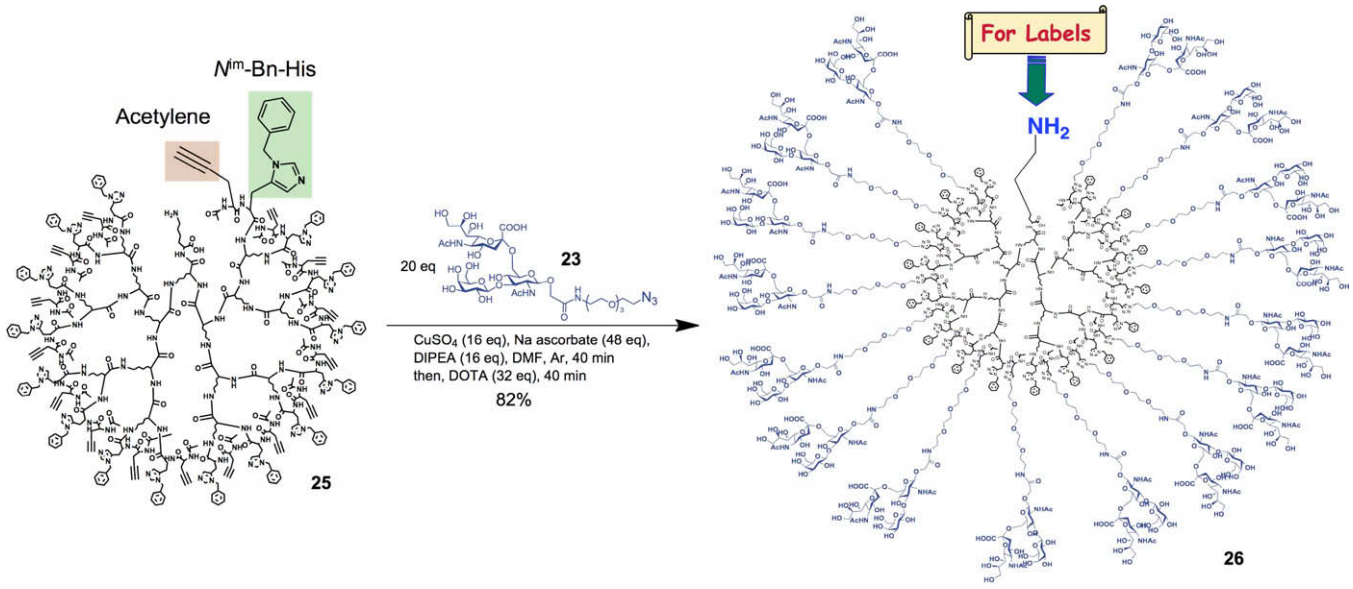
Alternatively, the allyl C=C double bond in **7** and **12** could also be used for the oxidative transformation to the carboxylic acid derivatives (Scheme 3); thus, the treatment of **7** and **12** with the $RuCl_3$ and $NaIO_4$ in CH_2Cl_2/MeCN/H_2O gave the carboxylic acids **18** and **19** in 57% and 67% yields. These acids were then coupled with the amine **20** in the presence of EDC and DMAP, and the alkaline hydrolysis of all esters yielded the azides **23** and **24** (69% and 92% in two steps), as the promising 'click' probes for the bioconjugate, for example, protein conjugation in preparing the antibodies, by Huisgen 1,3-dipolar cycloaddition reaction.[21]

In order to enhance the interaction of these glycan motifs with the target lectins,[1,11] the glycocluster probe[22] of the trisaccharide **26** was also developed (Scheme 4). Although many examples of glycoclusters to enhance the lectin-affinity[1,11] have been reported, the larger glycodendrimer of the complex glycan structures, such as the trisaccharide **23**, is quite rare. According to our 'self-activating' Huisgen 1,3-dipolar cycloaddition",[23] that utilizes the histidine as an internal 'Cu(I)'-coordinating ligand, the terminal acetylene of polylysine-based dendrimer template **25**, was reacted with azide-partner **23**, in the presence of copper sulfate, sodium ascorbate, and diisopropylethylamine at room temperature for 40 min.[22] After the residual copper ions were removed by chelation with DOTA and size-partitioning centrifugal filtration using Microcon® (MW = 10,000, Millipore), HPLC purification gave glycocluster **26** in 82%. Although the mother ion peaks of the glycocluster, a molecular weight of 22 kDa, could not be detected either by MALDI-TOF-MS and ESI-MS analyses, the size-partitioning gel filtration analysis and the representative signals in their ^{1}H NMR spectra confirmed the desired cluster. Since this cluster probe was designed to have an ε-amino group of lysine (Scheme 4), the cluster will be labeled either by biotin, metal chelators, or fluorescences through rapid 6π-azaelectrocyclization,[3,22] for various biological experiments.

In summary, we have synthesized the motifs of the *N*-glycan partial structure **1**, that is, tri- and tetrasaccharides of Galβ(1→3)[NeuAcα(2→6)]GlcNAcβ(1→2)Man, as the molecular probes for the biological evaluation. Key step is the quantitative and the completely α-selective sialylation of the disaccharide acceptor **4**, Galβ(1→3)GlcNTroc, with the C5-azide imidate **5**. Clusterization of the trisaccharide motif was also achieved by the 'self-activating click reaction'. It is noted that these probes could be efficiently be labeled by biotin and/or other fluorescence- or radioactive reporter groups through either cross metathesis, acylation, Cu(I)-mediated Huisgen [2+3]-cycloaddition, or the azaelectrocyclization to utilize the various biological techniques. Biological evaluation of these probes is now in progress in our laboratory.

2. Experimental

All commercially available reagents were used without further purification. Dichloromethane were refluxed over and distilled from CaH_2. Anhydrous DMF was purchased from Aldrich, and anhydrous THF was purchased from Kanto Chemicals, Tokyo. Preparative separation was usually performed by column chromatography on silica gel (FUJI silysia LTD, BW-200 and BW-300) and by thin layer chromatography on silica gel (Merck, 20 × 20 cm, Silica Gel 60 F_{254}, 1 mm). ^{1}H NMR spectra were recorded on either JEOL JNM-LA 400, 500, or 600 spectrometer and chemical shifts were represented as δ-values relative to the internal standard TMS. ESI-mass spectra were recorded on Applied Biosystems Marinar™ Biospectrometry Workstation. MALDI-TOF-mass spectra were measured on an SHIMADZU AXIMA-CFR mass spectrometer equipped with a nitrogen laser (λ = 337 nm).



Scheme 4. Synthesis of dendrimer-type cluster probe of trisaccharide.

2.1. Allyl 3-*O*-(2,3,4,6-*O*-tetra-benzoyl-β-D-galactopyranosyl)-6-*O*-(methyl-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-azido-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-α-D-glucopyranoside (6)

To a solution of sialic acid donor **5** (1.20 g, 1.80 mmol), disaccharide acceptor **4** (1.00 g, 1.02 mmol), and MS4 Å in dry propionitrile (10 mL) was added TMSOTf (74 μL, 0.40 mmol) at −78 °C under Ar atmosphere. After the mixture was stirred for 40 min at −78 °C, the reaction was quenched by triethylamine. After MS4 Å was removed by filtration, the filtrate was extracted with ethyl acetate, washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude product. The residue was purified by column chromatography on silica gel (toluene/EtOAc = 5:1) to afford the trisaccharide 6 as white solid (1.47 g, quantitative yield, α-isomer only): ^{1}H NMR (500 Hz, $CDCl_3$) δ 8.07 (t, 4H, J = 7.0 Hz, Bz), 7.91 (d, 2H, J = 7.5 Hz, Bz),7.71 (d, 2H, J = 7.0 Hz, Bz), 7.63 (t, 1H, J = 7.0 Hz, Bz), 7.57 (t, 1H, J = 7.0 Hz, Bz), 7.50 (t, 3H, J = 8.0 Hz, Bz), 7.45 (t, 2H, J = 8.0 Hz, Bz), 7.40 (t, 2H, J = 7.5 Hz, Bz), 7.37 (t, 2H, J = 7.5 Hz, Bz), 7.20 (t, 2H, J = 7.5 Hz, Bz), 5.97 (d, 1H, J = 3.1 Hz, Gal-H-1), 5.77–5.67 (m, 2H, $OCH_2CH{=}CH_2$, Glc-H-1), 5.62 (d, 1H, J = 8.4 Hz), 5.50 (s, 2H), 5.20 (ddd, 2H, J = 1.0 Hz, 10.3 Hz, 21.2 Hz), 5.01–4.99 (m, 1H), 4.93 (d, 1H, J = 8.0 Hz), 4.90–4.85 (m, 1H),4.75 (d, 1H, J = 3.5 Hz), 4.63 (dd, 1H, J = 5.5 Hz, 11.6 Hz), 4.55 (dd, 1H, J = 7.6 Hz, 11.6 Hz), 4.40 (t, 2H, J = 6.4 Hz), 4.32 (d, 1H, J = 13.0 Hz), 4.20 (dd, 1H, J = 3.5 Hz, 12.4 Hz), 4.11–4.04 (m, 2H), 3.90 (dd, 2H, J = 6.6 Hz, 12.7 Hz), 3.82 (d, 1H, J = 13.4 Hz), 3.74 (s, 3H, $COOCH_3$), 3.77–3.70 (m, 2H), 3.66 (dd, 1H, J = 1.8 Hz, 11.3 Hz), 3.58 (ddd, 1H, J = 1.9 Hz, 4.2 Hz, 5.8 Hz), 3.27 (t, 1H, J = 10.0 Hz), 2.77 (dd, 1H, J = 4.9 Hz, 12.9 Hz, H-3eq^{Neu}), 2.19, 2.13, 2.12, 2.01 (each s, 3H, $OCOCH_3$), 1.84 (dd, 1H, J = 12.9, 12.9 Hz, H-3ax^{Neu}); ESI-MS m/z calcd for $C_{64}H_{69}Cl_3N_4O_{27}Na$ $(M+Na)^+$ m/z 1453.31, found 1453.29.

2.2. Allyl 3-*O*-(2,3,4,6-*O*-tetra-benzoyl-β-D-galactopyranosyl)-6-*O*-(methyl-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-acetamide-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-4-*O*-acetyl-2-deoxy-2-acetamide-α-D-glucopyranoside (7)

To a solution of the trisaccharide **6** (30.0 mg, 21.0 μmol) obtained above in AcOH/THF (1:1, 2.0 mL) was added Zn/Cu powder at room temperature. After the mixture was stirred for 40 min at this temperature, the Zn/Cu powder was removed by filtration and the filtrate was concentrated in vacuo. The residue was extracted with AcOEt and the organic layer was washed with saturated aqueous $NaHCO_3$ solution and brine, dried over Na_2SO_4, and then concentrated in vacuo to give the intermediary diamine as a white solid, which was directly used for the double acetylation without further purification.

To a solution of the crude diamine obtained above in pyridine (2.0 mL) was added acetic anhydride (1.0 mL) at room temperature under Ar atmosphere. After the solution was stirred overnight at this temperature, the mixture was quenched by MeOH and concentrated in vacuo. The organic layer was extracted with ethyl acetate, washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude product. The residue was purified by preparative TLC on silica gel (EtOAc) to give *N*-acetyl derivative **7** as a white solid (18.2 mg, 64% for two steps): ^{1}H NMR (500 Hz $CDCl_3$) δ 8.11 (d, 1H, J = 0.2 Hz, Bz), 8.10 (d, 1H, J = 0.2 Hz, Bz), 8.07 (d, 1H, J = 0.2 Hz, Bz), 8.05 (d, 1H, J = 0.2 Hz, Bz), 7.91 (d, 1H, J = 0.2 Hz, Bz),7.89 (d, 1H, J = 0.2 Hz, Bz), 7.64 (t, 1H, J = 7.5 Hz, Bz), 7.59 (t, 1H, J = 7.5 Hz, Bz), 7.53 (t, 2H, J = 8.0 Hz, Bz), 7.49 (t, 1H, J = 7.5 Hz, Bz), 7.46 (t, 2H, J = 7.5 Hz, Bz), 7.41 (t, 1H, J = 7.5 Hz, Bz), 7.36 (t, 2H, J = 7.5 Hz, Bz), 7.23 (t, 2H, J = 7.5 Hz, Bz), 5.95 (d, 1H, J = 2.0 Hz, Gal-H-1), 5.77–5.67 (m, 1H, $OCH_2CH{=}CH_2$), 5.62 (d, 2H, J = 6.5 Hz), 5.50 (d, 1H, J = 9.0 Hz), 5.39–5.35 (m, 1H), 5.31 (dd, 1H, J = 2.0 Hz, 8.5 Hz), 5.24 (dd, 1H, J = 1.5 Hz, 17.0 Hz), 5.18 (dt, 3H, J = 2.5 Hz, 9.0 Hz), 5.01 (d, 1H, J = 6.5 Hz), 4.93–4.89 (m, 1H), 4.75 (d, 1H, J = 3.5 Hz), 4.65 (dd, 1H, J = 1.5 Hz, 11.5 Hz), 4.40 (dd, 1H, J = 7.0 Hz, 11.5 Hz), 4.34 (dd, 1H, J = 3.5 Hz, 10.5 Hz), 4.30 (dt, 2H, J = 3.0 Hz, 6.0 Hz), 4.10 (dd, 1H, J = 5.0 Hz, 13.5 Hz), 4.06–4.00 (m, 4H), 3.91–3.88 (m, 2H), 3.78 (s, 3H, $COOCH_3$), 3.44 (dd, 1H, J = 2.5 Hz, 11.0 Hz), 2.62 (dd, 1H, J = 5.0 Hz, 11.0 Hz, H-3eq^{Neu}), 2.15 (s, 3H, $NHCOCH_3$), 2.10, 2.05, 2.02, 1.89 and 1.71 (each s, 3H, $OCOCH_3$), 1.74 (dd, 1H, J = 11.0, 11.0 Hz, H-3ax^{Neu}); ESI-MS m/z calcd for $C_{67}H_{74}N_2O_{28}Na$ $(M+Na)^+$ m/z 1377.43, found 1377.33.

2.3. Allyl 2-*O*-[3-*O*-(2,3,4,6-*O*-tetra-benzoyl-β-D-galactopyranosyl)-6-*O*-(methyl-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-acetamide-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-4-*O*-acetyl-2-deoxy-2-(2,2,2-trichloroethoxycarbonylamino)-β-D-glucopyranosyl]-3-*O*-(4-azido-3-chlorobenzyl)-4,6-*O*-benzylidene-α-D-mannopyranoside (11)

To a solution of trisaccharide imidate **9** (50.6 mg, 31.2 μmol), mannose acceptor **10** (13.5 mg, 28.4 μmol), and MS4 Å in dry CH_2Cl_2 (2.0 mL) was added TMSOTf (1.5 μL, 8.50 μmol) at −40 °C

under Ar atmosphere. After the mixture was stirred for 1 h at −20 °C, the reaction was quenched by triethylamine at the same temperature. After MS4 Å was removed by filtration, the filtrate was extracted with ethyl acetate, washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude product. The residue was purified by column chromatography on silica gel ($CHCl_3$/acetone = 15:1) to afford the tetrasaccharide **11** as white solid (44.8 mg, 83%): ^{1}H NMR (500 Hz, $CDCl_3$) δ 10–8.06 (m, 6H), 8.00 (d, 2H, J = 7.0 Hz, Bz), 7.89 (dd, 1H, J = 8.5 Hz, 15.5 Hz, Bz), 7.77 (dd, 3H, J = 1.0 Hz, 5.0 Hz, Bz), 7.63 (t, 1H, J = 7.5 Hz, Bz), 7.57 (t, 1H, J = 7.5 Hz, Bz), 7.51 (t, 1H, J = 8.0 Hz, Bz), 7.47 (d, 1H, J = 4.0 Hz), 7.47 (s, 1H), 7.42 (t, 3H, J = 8.6 Hz), 7.37 (t, 5H, J = 4.4 Hz), 7.33 (d, 5H, J = 1.7 Hz), 7.22 (t, 3H, J = 8.3 Hz), 5.98 (d, 1H, J = 3.5 Hz, Gal-H-1), 5.89–5.78 (m, 2H, $OCH_2CH{=}CH_2$, Glc-H-1), 5.57 (dd, 1H, J = 3.6 Hz, 10.6 Hz), 5.49 (s, 1H), 5.46 (s, 1H), 5.46 (d, 1H, J = 6.1 Hz), 5.21 (t, 2H, J = 18.8 Hz), 4.98 (d, 1H, J = 9.0 Hz), 4.92–4.80 (m, 2H), 4.80–4.66 (m, 2H), 4.75 (s, 1H), 4.66–4.58 (m, 4H), 4.45 (d, 1H, J = 12.1 Hz), 4.41–4.32 (m, 3H), 4.31–4.24 (m, 1H), 4.16–4.08 (m, 7H), 4.00 (dd, 1H, J = 5.5 Hz, 11.2 Hz), 3.96–3.89 (m, 2H), 3.81 (d, 3H, J = 10.6 Hz), 3.74–3.65 (m, 4H), 3.66 (s, 3H, $COOCH_3$), 3.60 (t, 1H, J = 9.3 Hz), 3.45 (br s, 1H), 3.29–3.18 (m, 1H), 3.02–2.95 (m, 1H), 2.66 (dd, 1H, J = 4.9 Hz, 12.8 Hz, H-3eq^{Neu}), 2.63 (s, 3H, $NCOCH_3$),2.37–2.28 (m, 4H), 2.18, 2.12, 2.09, 2.04, 2.01, 1.88 (each s, 3H, $OCOCH_3$), 1.76 (dd, 1H, J = 12.4, 12.4 Hz, H-3ax^{Neu}); ESI-MS m/z calcd for $C_{88}H_{93}Cl_4N_5NaO_{34}$ $(M+Na+2H)^+$ m/z 1926.44, found 1926.38.

2.4. Allyl 2-*O*-[3-*O*-(2,3,4,6-*O*-tetra-benzoyl-β-D-galactopyranosyl)-6-*O*-(methyl-4,7,8,9-tetra-*O*-acetyl-3,5-dideoxy-5-acetamide-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-4-*O*-acetyl-2-deoxy-2-acetamide-β-D-glucopyranosyl]-3,4,6-*O*-tri-acetyl-α-D-mannopyranoside (12)

To a solution of tetrasaccharide **11** (65.8 mg, 34.5 μmol) obtained above in CH_2Cl_2 (5.0 mL) was added PPh_3 (8.4 mg, 41.5 μmol) at room temperature. After the mixture was stirred overnight at room temperature, DDQ (11.0 mg, 48.4 μmol), AcOH (31 μL), and H_2O (31 μL) were added, and the resulting solution was stirred overnight at room temperature. The mixture was diluted with ethyl acetate, washed with 5% aqueous L-ascorbic acid, saturated $NaHCO_3$ solution, and brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude alcohol, which was continuously deprotected without further purification.

To a solution of the crude alcohol obtained above in CH_2Cl_2 (5.0 mL) was added TFA (500 μL) and H_2O (500 μL) at 0 °C. After the mixture was stirred for 3 h at room temperature, the mixture was quenched by addition of saturated aqueous $NaHCO_3$ and extracted with EtOAc. The organic layer was washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude triol, which was immediately subjected to the Troc-deprotection without further purification.

To a solution of the crude triol obtained above in AcOH (5.0 mL) and THF (5.0 mL) was added Zn/Cu powder at room temperature. After the mixture was stirred for 40 min at this temperature, the metal powder was removed by filtration and the filtrate was concentrated in vacuo. The residue was extracted with AcOEt, and the organic layer was washed with saturated aqueous $NaHCO_3$ and brine, dried over Na_2SO_4, and then concentrated in vacuo to give the crude product, which was used for the acetylation without further purification.

To a solution of the crude product obtained above in pyridine (5.0 mL) was added acetic anhydride (5.0 mL) at room temperature under Ar atmosphere. After the solution was stirred overnight at this temperature, the reaction was quenched by MeOH and the mixture was directly concentrated in vacuo. The organic layer was extracted with ethyl acetate, washed with brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude product. The residue was purified by preparative TLC on silica gel ($CHCl_3$/MeOH = 20:1) to give **12** as a white solid (39.2 mg, 69% for 4 steps): ^{1}H NMR (500 Hz $CDCl_3$) δ 8.09 (t, 2H, J = 6.9 Hz, Bz), 8.04 (d, 2H, J = 7.5 Hz, Bz), 7.97 (dd, 2H, J = 7.5 Hz, 15.5 Hz, Bz), 7.75 (d, 2H, J = 7.5 Hz, Bz), 7.63 (t, 1H, J = 7.4 Hz, Bz), 7.57–740 (m, 9H, Bz), 7.22 (t, 2H, J = 7.5 Hz, Bz), 5.95 (d, 1H, J = 2.0 Hz, Gal-H-1), 5.94–5.86 (m, 1H, $OCH_2CH{=}CH_2$), 5.67–5.63 (m, 2H), 5.37–5.17 (m, 7H), 5.11–4.94 (m, 4H), 4.90–4.83 (m, 2H), 4.80–4.66 (m, 2H), 4.80–4.66 (m, 2H), 4.59 (dd, 1H, J = 6.3 Hz, 10.9 Hz), 4.37 (dd, 1H, J = 7.4 Hz, 10.9 Hz), 4.34–4.18 (m, 2H), 4.18–4.10 (m, 6H), 4.05–3.98 (m, 6H), 3.87 (dd, 1H, J = 4.6 Hz, 10.9 Hz), 3.79 (s, 3H, $COOCH_3$), 3.73 (s, 1H), 3.67 (s, 1H), 3.46 (dd, 1H, J = 6.3 Hz, 10.3 Hz), 3.32 (d, 1H, J = 10.9 Hz), 2.82 (t, 1H, J = 7.4 Hz), 2.63 (d, 1H, J = 2.3 Hz), 2.53 (dd, 1H, J = 4.6 Hz, 12.6 Hz, H-3eq^{Neu}), 2.37–2.28 (m, 4H), 2.12, 2.11, (s, 3H, $NHCOCH_3$), 2.07, 2.04, 2.02, 2.02, 2.00, 1.97, 1.89 and 1.83 (each s, 3H, $OCOCH_3$), 1.66 (dd, 1H, J = 12.6, 12.6 Hz, H-3ax^{Neu}); ESI-MS m/z calcd for $C_{79}H_{90}N_2O_{36}Na$ $(M+Na)^+$ m/z 1665.52, found 1665.55.

2.5. Representative procedure of cross metathesis with biotin derivative 13

To a solution of trisaccharide **7** (1.0 mg, 0.76 μmol) and biotin **13** (3.6 mg, 7.6 μmol) in dry CH_2Cl_2 (2.0 mL) was added Grubbs' second generation 'Ru' catalyst (500 μg, 76 nmol) at room temperature under Ar atmosphere. After the resulting solution was stirred overnight at 60 °C, the mixture was directly concentrated in vacuo to give the crude product. The residue was purified by preparative TLC on silica gel ($CHCl_3$/MeOH/H_2O = 8:2:0.2) to afford the trisaccharide **14** as white solid (1.4 mg, quantitative yield): ^{1}H NMR (500 Hz, $CDCl_3$; broad signals were observed over the whole range of the spectrum) δ 8.03–7.97 (m, 2H), 7.80 (d, 1H, J = 8.1 Hz), 7.68–7.62 (m, 2H), 7.55 (d, 1H, J = 15.9 Hz), 7.46–7.44 (m, 5H), 7.31–7.23 (m, 4H), 7.21–7.14 (m, 6H), 6.89 (br s, 1H), 6.43 (br m, 1H), 5.51 (br m, 1H), 5.31–5.24 (m, 3H), 5.08–4.78 (small broad peaks, 1H), 4.64 (br m, 1H), 4.41–4.35 (m, 1H), 4.24–4.20 (m, 3H), 4.16–4.06 (m, 1H), 3.98 (dd, 1H, J = 6.6, 6.6 Hz), 3.70 (br m), 3.60–3.35 (m, including –CO_2Me at δ 3.57, 3H), 3.08–3.03 (m, 1H), 2.83 (dd, 1H, J = 12.5, 4.4 Hz), 2.62 (d, 1H, J = 12.7 Hz), 2.37–2.21 (m), 2.14–2.10 (m), 2.074–2.066 (m), 2.00–1.87 (m), 1.81–1.19 (m), 0.84–0.76 (m); ESI-MS m/z calcd for $C_{86}H_{108}N_6O_{34}S$ $(M+2H)^+$ m/z 1800.66, found 1800.73. *Data for tetrasaccharide derivative* **15**: ^{1}H NMR (500 Hz, $CDCl_3$; broad signals were observed over the whole range of the spectrum) δ 8.13–8.04 (m, 4H), 8.01–7.95 (m, 2H), 7.78–7.63 (m, 4H), 7.61–7.54 (m, 4H), 7.50–7.37 (m, 4H), 7.36–7.13 (m, 2H), 6.84 (br m), 6.16 (dd, 1H, J = 15.0, 15.0 Hz), 5.97 (br m, 1H), 5.67 (br m, 1H), 5.42–5.28 (m, 4H), 5.19–5.12 (m, 2H), 5.07–4.98 (m, 1H), 4.95–4.88 (m, 1H), 4.82–4.72 (m), 4.68–4.58 (m, 1H), 4.54 (br m, 1H), 4.46–4.42 (m, 1H), 4.38–4.28 (m), 4.20–4.13 (m), 4.09–3.97 (m), 3.91–3.80 (m), 3.37–3.72 (m, including –CO_2Me at δ 3.67, 3H), 3.19 (br m, 1H), 3.07–2.86 (m), 2.79–2.73 (m, 1H), 2.60–2.54 (br m, 1H, H-3eq^{Neu}), 2.39–2.23 (m), 2.13–0.70 (m, including Ac-protons); MALID-TOF-MS m/z calcd for $C_{98}H_{122}N_6O_{42}SNa$ $(M+Na)^+$ m/z 2109.72, found 2109.5.

2.6. Representative procedure for conversion to azide derivatives

To a solution of trisaccharide **7** (59.7 mg, 42.7 μmol) and $NaIO_4$ (36.6 mg, 171 μmol) in CH_2Cl_2/MeCN/H_2O (2:2:3, 14.0 mL) was added $RuCl_3{\cdot}nH_2O$ (500 μg) at room temperature under Ar atmosphere. After the mixture was stirred for 2 h at this temperature, the reaction was diluted with H_2O, extracted with CH_2Cl_2, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude product. The residue was rapidly purified by preparative TLC on

silica gel ($CHCl_3$/MeOH/H_2O = 8:2:0.2) to afford **18** as white solid (34.7 mg, 57%), which was acylated after quickly identifying the carboxylic acid **18** by ESI-MS: *m*/*z* calcd for $C_{68}H_{74}N_2O_{31}Na$ $(M+Na)^+$ *m*/*z* 1437.42, found 1437.43.

To a solution of the carboxyl acid **18** obtained above (43.2 mg, 30.5 mol) and amine **20** (20.0 mg, 90.6 μmol) in CH_2Cl_2 (10 mL) was added EDC·HCl (5.9 mg, 30.5 μmol) and DMAP (4.1 mg, 33.6 μmol) at 0 °C under Ar atmosphere. After the mixture was stirred overnight at room temperature, the reaction was diluted with hexane, washed with H_2O, brine, dried over Na_2SO_4, filtered, and concentrated in vacuo to give the crude product. The residue was roughly purified by preparative TLC on silica gel ($CHCl_3$/MeOH = 10:1) to give the azide derivative **21** (33.8 mg, 69%, white solid), which without further purification was hydrolyzed after quickly identifying **21** by ESI-MS: *m*/*z* calcd for $C_{76}H_{91}N_6O_{33}$ $(M+H)^+$ *m*/*z* 1615.56, found 1615.35.

To a solution of **21** (5.0 mg, 3.09 μmol) obtained above in dry MeOH (1.0 mL) was added 100 mM aqueous NaOH (200 μL) at 0 °C. After the solution was stirred overnight at room temperature, the mixture was neutralized by ion-exchange resin (Dowex 50WX8, 50–100 mesh, H^+ form). The resin was removed by filtration and the filtrate was concentrated in vacuo. The residue was purified by reverse phase HPLC [column: Nacalai Tesque 5C_{18}-AR300, 4.6 × 250 mm; MeCN in H_2O containing 0.1% TFA (0–100% gradient over 30 min, 1 mL/min); UV detection at 250 nm] to afford the desired azide **23** as white solid (2.9 mg, quantitative yield): ^{1}H NMR (500 Hz CD_3OD) δ 7.80 (s 1H, COON*H*-PEG), 4.88 (1H, GlcNAc-H-1), 4.25 (d, 1H, *J* = 7.8 Hz, Gal-H-1), 4.08 (d, 1H, *J* = 15.2 Hz), 4.04 (dd, 1H, *J* = 3.3 Hz, 10.6 Hz, GlcNAc-H-3), 3.94–3.88 (m, 2H), 3.79 (m, characteristic vicinal protons of sugar hydroxyls and part of PEG-linker protons), 2.64 (m, 1H, H-3*eq*^{Neu}), 1.91 (s, 3H, $COCH_3$), 1.90 (s, 3H, $COCH_3$), 1.65 (t, 1H, *J* = 12.1 Hz, H-3*eq*^{Neu}); ESI-MS *m*/*z* calcd for $C_{35}H_{60}N_6O_{23}Na$ $(M+Na)^+$ *m*/*z* 955.36, found 955.53.

Data for tetrasaccharide carboxylic acid **19**: ^{1}H NMR (500 Hz, $CDCl_3$; broad signals were observed over the whole range of the spectrum) δ 8.09–7.92 (m, 6H), 7.74–7.68 (m, 3H), 7.63 (dd, 1H, *J* = 7.5, 7.5 Hz), 7.57–7.51 (m, 4H), 7.47–7.26 (m, 4H), 7.23–7.17 (m, 2H), 5.96–5.93 (m), 5.76–5.55 (m), 5.40–5.26 (m), 5.13–5.05 (m), 5.01–4.86 (m), 4.70–4.60 (m), 4.43–3.93 (m), 3.85–3.72 (m), 3.65 (s, 3H), 3.49–3.32 (m), 2.93 (br m, 1H), 2.59 (dd, 1H, *J* = 13.0, 5.0 Hz, H-3*eq*^{Neu}), 2.37–2.28 (m, 1H), 2.12–1.68 (m, including Ac-protons), 1.60 (br m, 1H, H-3*ax*^{Neu}); ESI-MS *m*/*z* calcd for $C_{78}H_{89}N_2O_{38}Na$ $(M+Na+H)^+$ *m*/*z* 1684.50, found 1684.48. *Data for tetrasaccharide azide* **22**: ^{1}H NMR (500 Hz $CDCl_3$) 8.10 (d, 2H, *J* = 6.5 Hz), 8.02 (d, 2H, *J* = 7.0 Hz, Bz), 7.98 (d, 2H, *J* = 7.0 Hz), 7.74 (d, 2H, *J* = 7.0 Hz), 7.63 (t, 1H, *J* = 7.0 Hz, Bz), 7.58–7.51 (m, 4H), 7.47 -7.40 (m, 4H), 7.22 (t, 3H, *J* = 8.0 Hz), 6.87 -6.83 (m, 1H), 5.95 (d, 1H, *J* = 3.5 Hz, Gal-H-1), 5.67–5.63 (m, 2H, $OCH_2CH{=}CH_2$, Glc-H-1), 5.34 (d, 1H, *J* = 6.5 Hz), 5.31 (s, 2H), 5.12–5.07 (m, 2H), 5.05–5.97 (m, 1H), 4.91–4.82 (m, 2H), 4.65–4.57 (m, 2H), 4.37 (dd, 1H, *J* = 7.5 Hz, 11.5 Hz), 4.28 (dd, 2H, *J* = 6.3 Hz, 13.2 Hz), 4.23 (d, 1H, *J* = 8.6 Hz), 4.14–4.09 (m, 2H), 4.05–3.95 (m, 4H), 3.89 (dd, 2H, *J* = 4.6 Hz, 10.9 Hz), 3.80 (s, 3H, $COOCH_3$), 3.69–3.60 (m, 11H, characteristic vicinal protons of sugar hydroxyls and part of PEG-linker protons), 3.53 (br s, 2H), 3.39 (t, 2H, *J* = 4.6 Hz), 3.33 (dd, 1H, *J* = 2.3 Hz, 10.9 Hz), 2.83 (dd, 1H, *J* = 8.0 Hz, 17.2 Hz), 2.53 (dd, 1H, *J* = 4.6 Hz, 12.6 Hz, H-3*eq*^{Neu}), 2.12, 2.10, (s, 3H, $NHCOCH_3$), 2.07, 2.04, 2.04, 2.01, 2.00, 1.97, 1.88 and 1.82 (each s, 3H, $OCOCH_3$), 1.69 (br s, H-3*ax*^{Neu}); ESI-MS *m*/*z* calcd for $C_{86}H_{105}N_6O_{40}$-Na $(M+Na+H)^+$ *m*/*z* 1884.63, found 1884.56.

2.7. Preparation of glycocluster 26

$CuSO_4$ (400 μg, 2.5 μmol), sodium L-ascorbate (1.5 mg, 7.4 μmol), and diisopropylethylamine (430 nL) were added to a solution of acetylene-containing polylysine dendrimer **25** (1.2 mg, 155 nmol) and trisaccharide azide **23** (2.9 mg, 3.1 μmol) in DMF (200 μL) and H_2O (600 μL) at room temperature under Ar atmosphere. After the mixture was stirred for 40 min at this temperature, DOTA (1,4,7,10-tetraazacyclodecane-1,4,7,10-tetraacetic acid, 5.0 mg, 12.4 μmol) was added, and the resulting solution was stirred for another 40 min. Low-molecule weight compounds were filtered using the Microcon® (centrifugal filter YM-10, 10,000 cut, Millipore), and the resulting aqueous solution was lipophilized to give glycocluster **26** as amorphous solid (2.9 mg, 82%). Cluster was then purified by reverse phase HPLC [column: Nacalai Tesque 5C_{18}-AR300, 4.6 × 250 mm; MeCN in H_2O containing 0.1% TFA (10–100% gradient over 40 min, 1 mL/min); UV detection at 250 nm; retention time: 12.2 min]. Molecular weight (22 kDa) was evaluated by gel filtration analysis [column: TSK-Gel G4000PW_{XL}, 7.8 × 300 mm; 0.1 M NaCl at pH 7.2, 1 mL/min; retention time: 10.4 min (reference glycoclusters of 12 kDa at 11.9 min, and 40 kDa at 9.9 min. Ref. 22)]: ^{1}H NMR (500 MHz, D_2O, HOD = δ 4.65) δ 8.69 (br s, 1H × 16, His-aromatic protons), 7.76 (s, 1H × 16, triazole, protons), 7.45–7.06 (m, 6H × 16, Bn- and His-aromatic protons), 5.24 (br s, 2H × 16, benzylic protons of Bn), 4.49 (s, 1H × 16), 4.38 (d, 2H × 16), 4.22–4.02 (m, 2H × 16 α-protons of Lys), 4.11–4.02 (m, 1H × 16), 4.03–3.21 (m, characteristic vicinal protons of sugar hydroxyls and part of PEG-linker protons), 3.21–3.06 and 3.05–2.78 (m, β-protons on His), 2.73–2.61 (m, H-3*eq*^{Neu}), 2.48–2.25 (m, β-protons of triazolic Ala), 1.95 and 1.84 (each s, Ac), 1.63 (t, *J* = 12.1 Hz, H-3*ax*^{Neu}), 1.15–1.00 and (m, part of acylpentane-linker protons), 1.88–1.75, 1.73–1.44, 1.15–1.00, and 0.95–0.72 (m, γ- and δ-protons of Lys).

Acknowledgments

G.B. and K.T. are equally contributors to the present work. This work was supported in parts by Grants-in-Aid for Scientific Research Nos. 19681024 and 19651095 from the Japan Society for the Promotion of Science, Collaborative Development of Innovative Seeds from Japan Science and Technology Agency (JST), New Energy and Industrial Technology Development Organization (NEDO, project ID: 07A01014a), as well as Molecular Imaging Research Program, Grants-in-Aid for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan.

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