Design and Synthesis of a Multivalent Heterobifunctional CD22 Ligand as a Potential Immunomodulator

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Abstract: We describe the design and synthesis of a trivalent CD22 ligand conjugated with a trivalent nitrophenol (ligand for decameric anti-nitrophenol immunoglobulin M). To generate such a bifunctional ligand, we designed and prepared two synthetic building blocks possessing different terminal functionalities. Coupling of these compounds afforded a trivalent azide-terminated scaffold. Ligation of the scaffold with an alkyne-terminated CD22 ligand by the use of click chemistry afforded the target trivalent cluster. We anticipate that our ligand will be valuable in a variety of applications, including targeting B cells and modulation of the humoral immune response.

Key words: bifunctional ligand, multivalent compounds, CD22, targeting, coupling, click chemistry

High-affinity protein ligands have wide applications in disease diagnosis, prevention and treatment. A promising strategy to arrive at such ligands is the synthesis of multivalent compounds. The design, synthesis and characterization of tailored multivalent ligands is currently a rapidly expanding frontier for novel pharmaceuticals.[1] Multivalent ligands provide a broad range of benefits and unique roles that are not achievable with monovalent ligands. This type of ligand could provide a dramatic amplification in avidity over monovalent ligands. They bind more strongly and selectively,[2] and induce receptor clustering (aggregation) on the cell surface.[3] In addition, multivalent interactions provide a strategy for controlling signal transduction pathways within cells.[4]

On the other hand, heterobifunctional ligands are a novel class of inhibitors that carry binding functionalities for the target receptor and an endogenous protein receptor.[5–11] The exceptional activity that is shown by this combination is achieved through supramolecular protein aggregation, an emerging concept that offers exciting possibilities in drug design.[3]

The use of a bifunctional ligand–antigen molecule to 'decorate' a target cell or microbe with an antigen has been proposed by several groups for targeting the immune response, and has been successfully demonstrated for targeting mammalian cells that contain the $\alpha_v\beta_3$ integrin[9] or folate receptor.[12]

B lymphocytes engage in signaling events that lead to immunity or tolerance. Both responses are mediated through antigen interactions with the B-cell antigen receptor (BCR).[2] CD22 (Siglec-2) is a B-cell-restricted glycoprotein and is a member of the Siglec family that is involved in signal transduction and modulation of cellular activation. It has been established as a target for immunotherapy of B-cell lymphomas and autoimmune diseases.[13] The carbohydrate ligand recognized by CD22 is the sequence Siaα2-6Gal on glycoproteins and glycolipids expressed by both the same B cell (*cis*-ligands) and cells that interact with B cells (e.g., T cells, *trans*-ligands). Interactions of CD22 with *cis*- or *trans*-ligands regulate aspects of B-cell activation, proliferation and development. CD22 exhibits low intrinsic affinity for its *in vivo* ligands (K_d = ~0.2 mM), and their abundance effectively masks the ligand-binding site.[14] Although CD22 is monovalent, it is concentrated in clathrin-rich microdomains and forms multimers in situ.[15] Thus, highly multivalent synthetic sialosides have shown improved inhibitory potency toward CD22.[16]

Paulson's group[17] recently linked the antigen nitrophenol to a modified trisaccharide ligand for CD22. The resulting bifunctional molecule was able to form complexes with decameric anti-nitrophenol immunoglobulin M (anti-NP IgM) and form a multivalent ligand for CD22. The multivalent form of a potent ligand is expected to overcome the *cis* and *trans* interactions of endogenous ligands. In effect, these complexes efficiently bind to the B-cell surface, and induce IgM-mediated complement activation and killing of the cells, offering an attractive therapeutic approach for the treatment of B-cell leukemia. Accordingly, CD22 can be targeted in B-cell-depletion therapy, which is now recognized to have a therapeutic benefit for B-cell leukemia as well as a number of chronic inflammatory diseases such as rheumatoid arthritis and systemic lupus erythematosus.[18]

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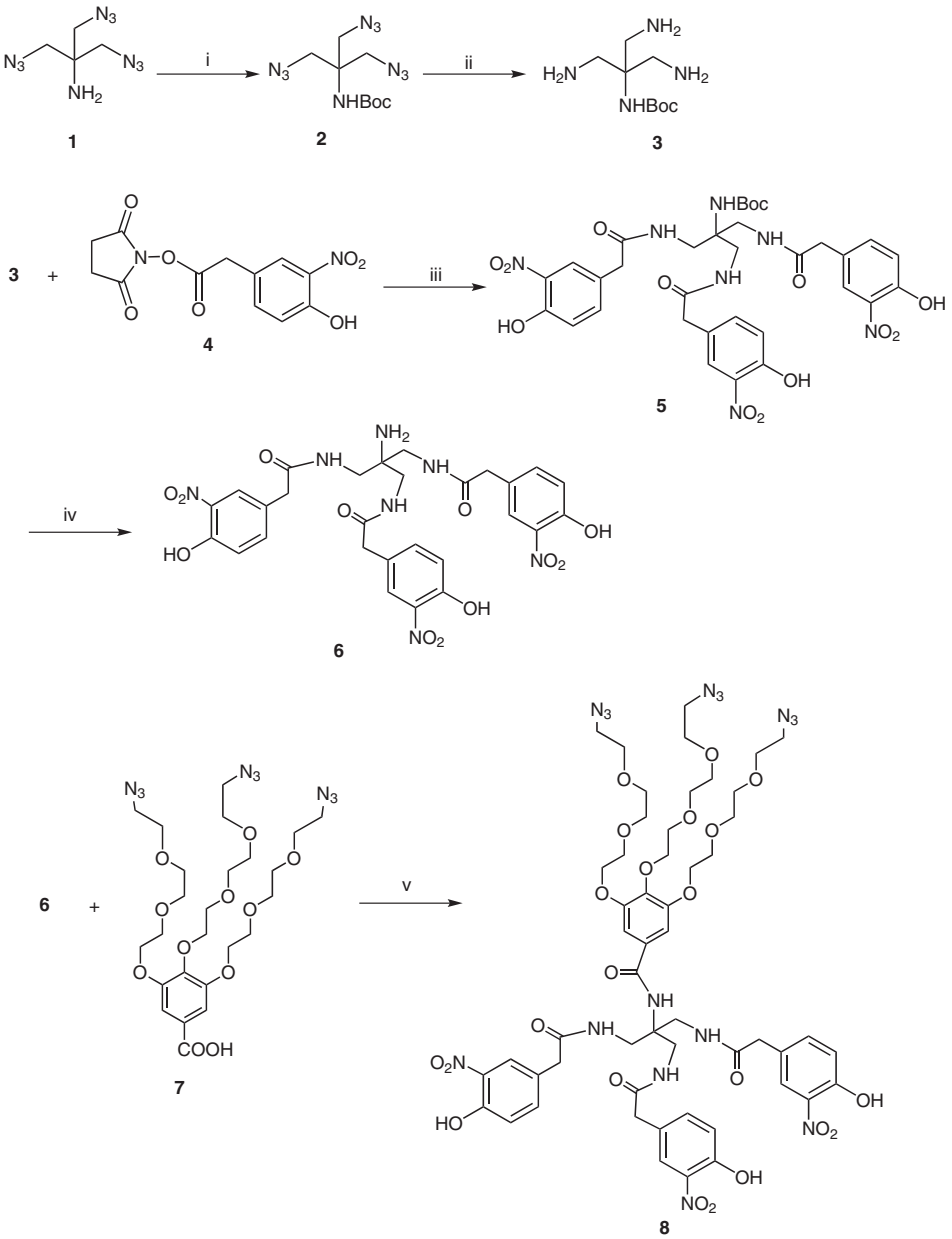
Multivalent antigen–CD22 ligand complexes also inhibit B-cell activation probably by activating CD22-mediated signal inhibition. Such complexes inhibit B-cell signaling in B cells reactive to the antigen, and suppress antibody production to the antigen,[8,10] suggesting that these complexes can be used for antigen-specific immune suppression therapies; however, these complexes are highly multimeric ($n > 100$) and therefore too large in size to achieve efficient binding to CD22 on B cells. To overcome this problem by using a synthetic compound that binds to CD22 with high affinity,[19,20] we report herein the synthesis of an oligovalent bifunctional ligand that would simultaneously bind to CD22 and anti-nitrophenol immunoglobulins. This high-affinity compound comprises the preferred glycan sequence recognized by CD22 (Neu5Gcα2-6Gal) with a 9-[(4′-hydroxy-1,1′-biphenyl-4-yl)acetamido] substituent on the sialic acid. This compound was chosen based on our extensive SAR study on CD22 ligands that revealed its high binding affinity for CD22.[19]

There are three important components of the designed ligand: (i) NP as an antigen; (ii) a potent CD22 ligand; (iii) a linker that connects and separates the antigen from the ligand. Importantly, the effect of linker structure and length on bifunctional-ligand-driven assembly of IgM–CD22 complexes on native B cells has been previously investigated and it was found that complex formation is tolerant to changes in the linker structure and length.[17]

The synthetic strategy utilized in our work was based on the preparation of two branched building blocks **6** and **7** possessing different terminal functionalities (Scheme 1).



Scheme 1 *Reagents and conditions*: (i) Boc_2O, EtOH, r.t., 8 h, 90%; (ii) H_2, Pd/C, EtOH, r.t., 8 h, 97%; (iii) Et_3N, MeOH (pH 9), r.t., 36 h, 77.5%; (iv) TFA, CH_2Cl_2, H_2O, r.t., 8 h, 95%; (v) EDCl, HOBt, CH_2Cl_2, 75%.

Both **6** and **7** are hydrophilic and could be readily prepared from inexpensive starting materials; namely, tris(hydroxymethyl)aminomethane (TRIS), gallic acid and triethylene glycol.

As shown in Scheme 1, building block **6** was obtained in six steps from TRIS which is an interesting molecule that allows the attachment of four identical or different groups. Hence, it serves as a versatile scaffold for the construction of highly branched structures.[21–24] Thus, chlorination[25] of TRIS followed by azidation[23] gave tris(azidomethyl)methylamine (**1**).[23] Amine **1** was Boc protected to provide **2** and the three azido groups were reduced by catalytic hydrogenation to give the triamine **3** in 97% crude yield. The resulting triamine **3** was acylated by coupling with 3.6 equivalents of succinimido (4-hydroxy-3-nitrophenyl)acetate (**4**)[26] in the presence of triethylamine in methanol to give compound **5** in 77.5% yield. Deprotection of **5** (removal of Boc) with trifluoroacetic acid in dichloromethane, followed by the addition of 1 M sodium carbonate, afforded the key building block **6** in 95% yield.

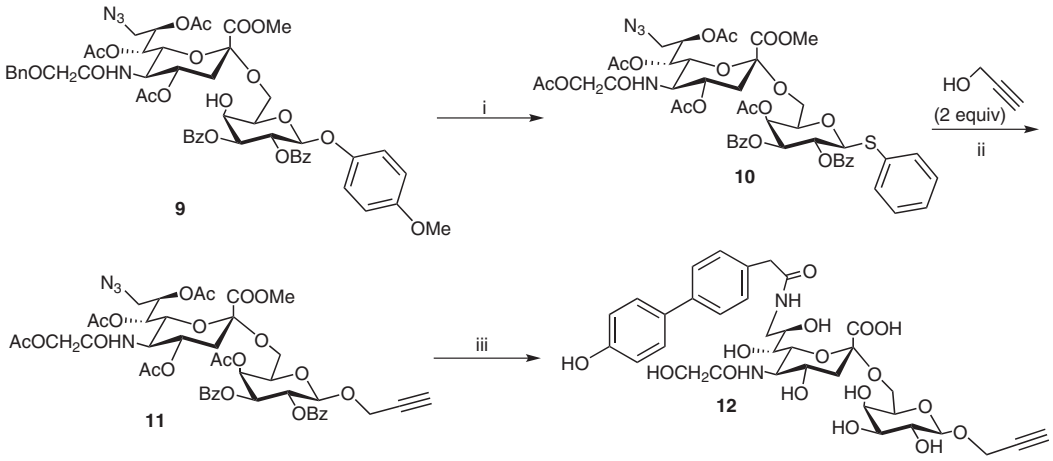
On the other hand, building block **7**[27,28] is composed of gallic acid (3,4,5-trihydroxybenzoic acid) as the trivalent core and triethylene glycol as the hydrophilic spacer. We chose the spacer to ensure water solubility of the final compound, to counteract the hydrophobic effect of the aromatic backbone, to reduce nonspecific binding, to impart a degree of flexibility and to provide sufficient spacing to allow the carbohydrate moieties to be readily accessible by receptor sites.[27,28] Thus, gallic acid was esterified, alkylated with the appropriate monoazido tosylate, then deesterified to afford the triazide-terminated gallic acid–triethylene glycol core **7**, as previously reported.[27,28]

Several strategies have been reported to anchor carbohydrates to clusters; however, it has been found advantageous to attach the carbohydrate residues after buildup of the cluster core.[28] Thereby, unprotected carbohydrate is introduced avoiding tedious final deprotection steps and leading to more efficient couplings for steric reasons.[27] In our approach, we resolved to locate the azide and alkyne functional groups on the cluster core and the carbohydrate partner, respectively. Azide-terminated dendrimers were preferred to those incorporating terminal alkynes because of the potential bias of the latter for copper(II)-catalyzed intradendritic oxidative coupling.[27,29] In addition, the required *O*-alkynyl carbohydrates are easily accessible.

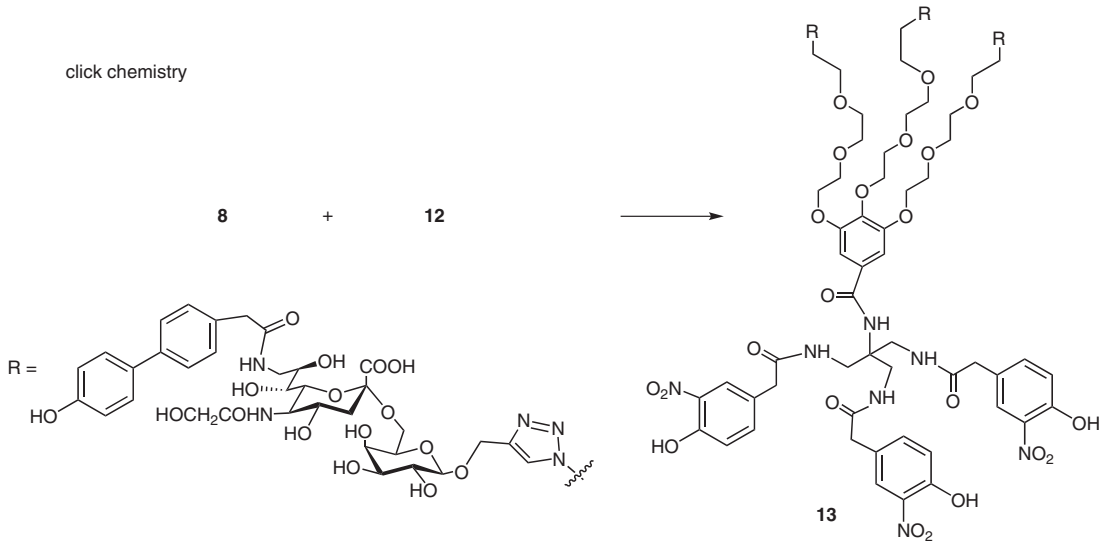
With a reliable synthesis of the two precursors **6** and **7** in hand, azide-terminated scaffold **8**, carrying a hydrolytically stable amide bond at the focal point, was synthesized. Thus, scaffold **8** was obtained from amine **6** and acid **7** through an amide linkage (EDCl, HOBt, CH_2Cl_2) in 75% yield (Scheme 1). The structure of **8** was confirmed by IR spectroscopy, which showed characteristic peaks for azide (2106 cm^{-1}), amidic carbonyl (1680 cm^{-1}) and nitro (1530 and 1245 cm^{-1}). The ^1H NMR spectrum of **8** showed the following key signals: a singlet at $\delta = 6.95$ ppm (2 H) for the gallic acid residue; three signals, each of 3 H, that correspond to the aromatic protons of the NP moiety; additionally, a signal (3 H) for phenolic OH. The HRMS data showed the presence of a signal at m/z = 1301.4435, identical to the calculated value for the $[M + Na]^+$ adduct.

Copper(I)-catalyzed azide–alkyne [3+2] cycloaddition,[30] the most efficient among the click reactions[31] as it proceeds in high yields, under aqueous conditions and with complete regioselectivity, was proposed for ligation of the azide-terminated scaffold **8** with the alkyne-derived unprotected sialoside **12**.

Compound **12** is the alkynated form of one of the most potent CD22 ligands.[19] We have described the synthesis of this compound in a recent communication.[20] Thus, sialoside **12** was synthesized starting from compound **9** (Scheme 2).[19] Selective debenzylation of **9** with the simultaneous conversion of the *p*-methoxyphenyl residue into the corresponding phenyl thioglycoside was achieved in one step by the Hanessian reaction method.[20] Direct acetylation of the resulting product in the presence of 4-(dimethylamino)pyridine afforded compound **10** in 87% yield (two steps). For glycosylation of phenyl thioglyco-



Scheme 2 *Reagents and conditions*: (i) (a) $PhSSiMe_3$ (10 equiv), ZnI_2 (5 equiv), Bu_4NI (3 equiv), DCE, 60 °C, 36 h; (b) Ac_2O, DMAP, pyridine, 87% (two steps); (ii) BSP, TTBP, Tf_2O, CH_2Cl_2, 4 Å MS, –60 to 0 °C, 8 h, 70%; (iii) (a) LiOH, EtOH–H_2O; (b) Me_3P, MeOH–H_2O, r.t., 16 h; (c) succinimido ester, $NaHCO_3$, MeCN–H_2O, 50% (three steps).



Scheme 3 *Reagents and conditions*: $CuSO_4$, sodium ascorbate, H_2O–*t*-BuOH (1:1), r.t., 48 h, 48.5%.

side donor **10** with the propargyl alcohol acceptor, we tried the promoter system 1-(phenylsulfinyl)piperidine (also known as 1-benzenesulfinyl piperidine, BSP)/trifluoromethanesulfonic anhydride, a potent thiophilic activator system developed by Crich and co-workers.[32] This method has been demonstrated to afford high glycosylation yields and excellent stereoselectivity through the formation of glycosyl triflate intermediates. Fortunately, clean reaction was observed using the reported protocol; thioglycoside **10** was treated with 1-(phenylsulfinyl)piperidine, 2,4,6-tri-*tert*-butylpyrimidine (TTBP) and molecular sieves in dichloromethane at –60 °C, followed by the addition of trifluoromethanesulfonic anhydride and a solution of propargyl alcohol in dichloromethane. The propargyl glycoside **11** was obtained in good yield (70%) and excellent stereoselectivity; only a trace amount of the α-glycoside was detected in the ^{1}H NMR spectrum of the crude product, and none could be isolated. The stereochemistry of the new glycoside was confirmed as β from the ^{1}H NMR spectrum [δ = 5.03 (d, $J_{1,2}$ = 8.24 Hz, $H1_{Gal}$)]. Complete deprotection of glycoside **11** by hydrolysis with lithium hydroxide followed by chemoselective reduction of azide using trimethylphosphine in aqueous methanol afforded the corresponding free amine, which was directly acylated with succinimido (4′-hydroxy-1,1′-biphenyl-4-yl)acetate[19] to provide **12** in 50% yield (three steps).

Conjugation of scaffold **8** to sialoside **12** by click chemistry afforded the target cluster **13** (Scheme 3) which was isolated and purified by Sephadex® then reversed-phase column chromatography; this gave a yellow solid (9.5 mg, 48.5% yield) which is freely soluble in water.

Analysis of the ^{1}H NMR spectrum of cluster **13** showed the presence of signals for the triazole [δ = 7.99 (br s, 3 H, 3 triazole ring system)], the glycosidic CH_2 between the triazole ring and the galactose residue [δ = 4.51 (m, 6 H, glycosidic CH_2)], and the sialoside-associated signals [δ = 4.35 (br d, 3 H, 3 H_{1a}), 2.80 (br d, 3 H, 3 $H_{3b\text{-}eq}$), 1.67 (br t, 3 H, 3 $H_{3b\text{-}ax}$)], in a consistent ratio. The completion of the substitution and the purity of the resulting sialylated product were confirmed by stage-discriminated spectral correlation of energy-resolved mass spectrometry (SDC of ERMS). This technique has been recently described as a mild technique for detection of the identity and purity of glycans.[33] The structure of compound **13** was confirmed from its *m*/*z* value and from the fragmentation pattern under collision-induced dissociation conditions which showed the presence of a signal at *m*/*z* = 1159.6, matching well the calculated value of *m*/*z* = 1159.4 for $[M - H]^{3-}$. Another signal at *m*/*z* = 869.5 matches well the calculated value of *m*/*z* = 869.3 for $[M - 3\ H]^{4-}$. Fragmentation at the labile anomeric position of the sialic acid residue can also be detected (see Supporting Information, Figures 1 and 2). The biological activity of compound **13** is under investigation.

In conclusion, heterobifunctional trivalent cluster **13** that displays, in addition to an antigen (nitrophenol, NP), a targeting moiety that recognizes CD22 was constructed to serve as a multivalent CD22 ligand for B-cell targeting and modulation of the immune response. The synthetic strategy utilized in this work was based on the preparation of two branched building blocks **6** and **7** possessing different terminal functionalities. Coupling of compounds **6** and **7** afforded the trivalent azide-terminated scaffold **8**. Ligation of scaffold **8** with alkyne-terminated CD22 ligand **12** by the use of click chemistry afforded the target trivalent cluster **13**. Bifunctional conjugates of this type could facilitate the development of new methods for modulating humoral immune responses in an antigen-specific manner.

Solvents and reagents were used as received from commercial distributors without further purification. Anhydrous reactions were conducted under an argon atmosphere. ^{1}H and ^{13}C NMR spectra were recorded on a JEOL JNM-EX-400 (400 MHz), JEOL JNM-EX-500 (500 MHz) or JEOL JNM-ECA-600 (600 MHz) instrument. Chemical shifts are expressed in ppm (δ) relative to the signal for TMS. Galactose protons are assigned ‘a’, while those of sialic

acid are assigned 'b'. High-resolution mass spectrometry (HRMS) was performed with a Bruker Daltonics micrOTOF (ESI-TOF) mass spectrometer. The multivalent ligand **13** was analyzed using a quadrupole ion trap mass spectrometer (QIT-MS) coupled with an electrospray interface (Bruker Esquire 3000 plus, Bruker Daltonik GmbH, Bremen, Germany). TLC analysis was carried out on Merck TLC glass plates (silica gel 60 F_{254}) and compounds were visualized by exposure to UV light and/or by charring with 10% H_2SO_4 in EtOH. Flash column chromatography on silica gel (Fuji Silysia Co., 80 and 300 mesh) or Sephadex® (Pharmacia LH-20) was performed with the solvent systems (v/v) specified. The quantity of silica gel was usually estimated as 100- to 150-fold weight of the sample to be charged. Reversed-phase silica gel (Wakogel® 50 C18) was purchased from Wako Pure Chemical Industries, Ltd. HPLC grade H_2O and MeOH (Wako) were used for purification of the final compounds. Solvent systems for chromatography are specified in v/v. All evaporations and concentrations were carried out under reduced pressure.

1,1,1-Tris(azidomethyl)-*N*-(*tert*-butoxycarbonyl)methylamine (2)

A soln of tris(azidomethyl)methylamine[23] (**1**; 7.20 g, 36.66 mmol) in EtOH (100 mL) was added to a soln of Boc_2O (8 g, 36.66 mmol) in EtOH (100 mL). The reaction mixture was stirred at r.t. for 8 h, then concentrated to dryness to provide a white solid which was recrystallized (H_2O) to give **2**; yield: 9.77 g (90%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 4.70 (br s, 1 H, NH), 3.62 (s, 6 H, 3 CH_2), 1.45 (s, 9 H, 3 CH_3).

1,1,1-Tris(aminomethyl)-*N*-(*tert*-butoxycarbonyl)methylamine (3)

A round-bottom flask equipped with a stirrer bar was charged with triazide **2** (1.50 g, 5.06 mmol), EtOH (100 mL) and 10% Pd/C (1 g). The resulting mixture was stirred under H_2 at r.t. for 8 h; then, the mixture was filtered through Celite® and the filtrate was concentrated to give triamine **3** as a colorless oil; yield: 1.07 g (97%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 5.68 (br s, 1 H, NH), 2.89 (br s, 6 H, NH_2), 2.84 (s, 6 H, CH_2), 1.43 (s, 9 H, 3 CH_3).

***N*-(*tert*-Butoxycarbonyl)-1,1,1-tris[(4-hydroxy-3-nitrophenyl)acetamidomethyl]methylamine (5)**

To a soln of triamine **3** (100 mg, 0.46 mmol) in MeOH (15 mL) was added succinimido (4-hydroxy-3-nitrophenyl)acetate[26] (**4**; 487 mg, 1.65 mmol). The solution was kept basic with the addition of Et_3N and stirred at r.t. for 36 h. The volatiles were evaporated and the residue was purified by silica gel column chromatography (MeOH–$CHCl_3$, 2:98) to afford **5** as an orange-colored solid; yield: 268 mg (77.5%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.00 (d, *J* = 1.8 Hz, 3 H, ArH), 7.51 (dd, *J* = 8.3, 1.8 Hz, 3 H, ArH), 7.43 (br s, 3 H, 3 OH), 7.10 (d, *J* = 8.3 Hz, 3 H, ArH), 5.55 (br s, 1 H, NHCOO), 3.54 (s, 6 H, ArCH_2CO), 3.45 (t, *J* = 6.2 Hz, 3 H, N*H*CH_2), 3.28 (d, *J* = 6.2 Hz, 6 H, NHCH_2), 1.32 (s, 9 H, 3 CH_3).

HRMS: *m*/*z* [M + Na]$^+$ calcd for $C_{33}H_{37}N_7O_{14}Na$: 778.2296; found: 778.2296.

1,1,1-Tris[(4-hydroxy-3-nitrophenyl)acetamidomethyl]methylamine (6)

A TFA–H_2O mixture (3:1, 2 mL) was added to a soln of **5** (250 mg, 0.33 mmol) in CH_2Cl_2 (1 mL). The reaction mixture was stirred for 8 h, then treated with 1 M Na_2CO_3 (5 mL); the aqueous phase was extracted with CH_2Cl_2 (5 × 5 mL). The combined organic layers were dried ($MgSO_4$), filtered and then evaporated to dryness to give **6** as an orange-colored solid; yield: 206 mg (95%).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.98 (d, *J* = 2.0 Hz, 3 H, ArH), 7.58 (br s, 3 H, 3 OH), 7.45 (dd, *J* = 8.2, 2.0 Hz, 3 H, ArH), 7.10 (d, *J* = 8.2 Hz, 3 H, ArH), 3.45 (m, 9 H, ArCH_2CO + N*H*CH_2), 3.10 (m, 8 H, NHCH_2, NH_2).

HRMS: *m*/*z* [M + H]$^+$ calcd for $C_{29}H_{30}N_7O_{12}$: 656.1952; found: 656.1952.

3,4,5-Tris{2-[2-(2-azidoethoxy)ethoxy]ethoxy}-*N*-{1,1,1-tris[(4-hydroxy-3-nitrophenyl)acetamidomethyl]methyl}benzamide (8)

A soln of acid **7**[27,28] (179.6 mg, 0.28 mmol) in anhyd CH_2Cl_2 (3 mL) was cooled to 0 °C. To this mixture was added HOBt (42.5 mg, 0.28 mmol) and EDCl (53.5 mg, 0.28 mmol) at 0 °C and the mixture was stirred for 30 min. To this was added amine **6** (113 mg, 0.172 mmol) while stirring, and the reaction mixture was warmed to r.t. Stirring was continued for 24 h under argon. Then, the mixture was diluted with CH_2Cl_2 (20 mL), washed with brine (3 × 15 mL), dried (Na_2SO_4) and concentrated under reduced pressure. Purification of the residue by silica gel chromatography (0.5–1% MeOH in $CHCl_3$) afforded **8** as an orange-colored solid; yield: 165 mg (75%).

IR (KBr): 2106 (azide), 1680 (amidic carbonyl), 1530 and 1245 cm^{-1} (nitro).

^{1}H NMR (400 MHz, $CDCl_3$): δ = 8.02 (d, *J* = 2.0 Hz, 3 H, ArH), 7.53–7.45 (m, 6 H, 3 OH + 3 ArH), 7.10 (d, *J* = 8.2 Hz, 3 H, ArH), 6.95 (s, 2 H, H of gallic residue), 4.23–4.13 (m, 7 H, 3 CH_2N_3, CONH), 3.89–3.55 (m, 33 H, 3 ArCH_2CO + 3 N*H*CH_2, 12 CH_2O), 3.40–3.36 (m, 12 H, 3 NHCH_2, 3 CH_2O).

HRMS: *m*/*z* [M + Na]$^+$ calcd for $C_{53}H_{66}N_{16}O_{22}Na$: 1301.4435; found: 1301.4435.

Phenyl (Methyl 5-(Acetoxyacetamido)-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→6)-4-*O*-acetyl-2,3-di-*O*-benzoyl-1-thio-β-D-galactopyranoside (10)

To a mixture of anhyd ZnI_2 (0.85 g, 2.7 mmol, 5 equiv) and Bu_4NI (0.6 g, 1.62 mmol, 3 equiv) was added a soln of compound **9**[19] (0.57 g, 0.54 mmol) in DCE (10 mL) followed by $PhSSiMe_3$ (0.9 mL, 5.4 mmol, 10 equiv). The mixture was stirred at 60 °C for 36 h until no starting material remained by TLC analysis (MeOH–$CHCl_3$, 2:98). The reaction mixture was cooled, diluted with CH_2Cl_2 (30 mL), washed with H_2O (30 mL) and dried. The crude product, obtained after solvent removal, was acetylated with Ac_2O–pyridine (1:1, 8 mL) in the presence of DMAP. After completion of the reaction, a few drops of H_2O were added. The reaction mixture was concentrated in vacuo and extracted with $CHCl_3$ (50 mL), washed with dil. HCl (50 mL) and brine (50 mL). The organic extract was dried (Na_2SO_4) and then evaporated. The residue was purified by column chromatography (MeOH–$CHCl_3$, 0.75:99.25) to give **10**; yield: 0.49 g (87%).

R_f = 0.2 (MeOH–$CHCl_3$, 1:99).

^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.97 (d, *J* = 7.5 Hz, 2 H, ArH), 7.85 (d, *J* = 7.5 Hz, 2 H, ArH), 7.57–7.31 (m, 11 H, ArH), 5.88 (d, *J* = 13.1 Hz, 1 H, NH), 5.72–5.69 (m, 2 H, H_{2a}, H_{4a}), 5.46 (br d, *J* = 10.3 Hz, 1 H, H_{3a}), 5.35 (m, 1 H, H_{8b}), 5.26 (d, *J* = 7.5 Hz, 1 H, H_{7b}), 5.07 (d, *J* = 10.3 Hz, 1 H, H_{1a}), 4.93 (m, 1 H, H_{4b}), 4.61 (d, *J* = 15.4 Hz, 1 H, AcOCH_2CO), 4.29 (d, *J* = 15.4 Hz, 1 H, AcOCH_2CO), 4.17–4.09 (m, 3 H, $H_{6a'}$, H_{5b}, H_{6b}), 3.94–3.91 (m, 1 H, H_{5a}), 3.82 (s, 3 H, $COOCH_3$), 3.74 (br d, *J* = 13.7 Hz, 1 H, $H_{6a''}$), 3.50 (br q, 1 H, $H_{9b'}$), 3.33 (br q, 1 H, $H_{9b''}$), 2.60 (br d, *J* = 12.2 Hz, 1 H, $H_{3b\text{-}eq}$), 2.24–2.02 (m, 15 H, 5 OAc), 1.97 (t, *J* = 12.4 Hz, 1 H, $H_{3b\text{-}ax}$).

^{13}C NMR (150 MHz, $CDCl_3$): δ = 171.0, 170.5, 169.9, 169.6, 167.8, 167.6, 165.3, 133.3, 133.0, 132.3, 129.8, 129.6, 129.5, 129.3, 128.9, 128.8, 128.4, 128.3, 127.9, 99.0, 86.5, 73.0, 72.9, 69.9, 68.0, 67.9, 67.8, 67.7, 62.7, 53.0, 51.0, 49.3, 38.0, 29.6, 21.0, 20.7, 20.6, 20.5.

HRMS: *m/z* [M + Na]$^+$ calcd for $C_{48}H_{52}N_4O_{20}NaS$: 1059.2793; found: 1059.2793.

2-Propynyl (Methyl 5-(Acetoxyacetamido)-4,7,8-tri-*O*-acetyl-9-azido-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonate)-(2→6)-4-*O*-acetyl-2,3-di-*O*-benzoyl-β-D-galactopyranoside (11)

To a stirred soln containing the thioglycoside **10** (165 mg, 0.16 mmol), 1-(phenylsulfinyl)piperidine (BSP; 50.4 mg, 0.24 mmol, 1.5 equiv), 2,4,6-tri-*tert*-butylpyrimidine (TTBP; 79.0 mg, 0.32 mmol, 2 equiv) and activated 4 Å powdered molecular sieves (300 mg) in CH_2Cl_2 (4 mL), at –60 °C under an argon atmosphere, was slowly added Tf_2O (29 μL, 0.17 mmol, 1.1 equiv). The resulting yellow mixture was stirred at this temperature for 30 min; then, a soln of 2-propynol (18 μL, 0.32 mmol, 2 equiv) in CH_2Cl_2 (2 mL) was added. The reaction mixture was stirred at –60 °C for 5 min and then warmed gradually to 0 °C (for 6 h), filtered, washed with sat. aq $NaHCO_3$ (5 mL) and then brine (5 mL), dried and concentrated under reduced pressure. The residue was purified by column chromatography (MeOH–$CHCl_3$, 0.75:99.25) to give **11**; yield: 109.5 mg (70%).

R_f = 0.25 (MeOH–$CHCl_3$, 1:99).

IR (KBr): 2350 (C≡C), 2150 cm^{-1} (azide).

^{1}H NMR (600 MHz, $CDCl_3$): δ = 7.98 (d, *J* = 7.5 Hz, 2 H, ArH), 7.88 (d, *J* = 7.5 Hz, 2 H, ArH), 7.49 (m, 2 H, ArH), 7.38–7.34 (m, 4 H, ArH), 5.87 (d, *J* = 9.8 Hz, 1 H, NH), 5.71–5.65 (m, 2 H, H_{2a}, H_{4a}), 5.44 (dd, *J* = 10.0, 3.4 Hz, 1 H, H_{3a}), 5.33 (m, 1 H, H_{8b}), 5.27 (d, *J* = 9.6 Hz, 1 H, H_{7b}), 5.03 (d, *J* = 8.2 Hz, 1 H, H_{1a}), 4.91 (m, 1 H, H_{4b}), 4.61 (d, *J* = 15.1 Hz, 1 H, AcOCH_2CO), 4.47 (dd, *J* = 15.8, 2.5 Hz, 1 H, propargylic CH_2), 4.41 (dd, *J* = 15.8, 2.5 Hz, 1 H, propargylic CH_2), 4.30 (d, *J* = 15.1 Hz, 1 H, AcOCH_2CO), 4.16–4.08 (m, 3 H, $H_{6a'}$, H_{5b}, H_{6b}), 3.88 (m, 1 H, H_{5a}), 3.82 (s, 3 H, $COOCH_3$), 3.66 (br d, *J* = 11.0 Hz, 1 H, $H_{6a''}$), 3.48 (dd, *J* = 6.2, 13.8 Hz, 1 H, $H_{9b'}$), 3.32 (dd, *J* = 6.2, 13.8 Hz, 1 H, $H_{9b''}$), 2.57 (dd, *J* = 4.8, 13.0 Hz, 1 H, $H_{3b\text{-}eq}$), 2.40 (t, *J* = 2.4 Hz, alkynyl H), 2.19–2.13 (m, 12 H, 4 OAc), 2.00 (s, 3 H, OAc), 1.97 (t, *J* = 13.0 Hz, 1 H, $H_{3b\text{-}ax}$).

HRMS: *m/z* [M + Na]$^+$ calcd for $C_{45}H_{50}N_4O_{21}Na$: 1005.2865; found: 1005.2865.

2-Propynyl (5-Glycolamido-9-[(4′-hydroxy-1,1′-biphenyl-4-yl)acetamido]-3,5,9-trideoxy-D-*glycero*-α-D-*galacto*-2-nonulopyranosylonic Acid)-(2→6)-β-D-galactopyranoside (12)

To a soln of glycoside **11** (70 mg, 0.07 mmol) in EtOH (3 mL) was added a soln of LiOH·H_2O (36 mg, 0.85 mmol, 12 equiv) in H_2O (1 mL). After being stirred at r.t. for 8 h, the reaction mixture was treated with acidic resin (DOWEX® 50, H$^+$) and the suspension was filtered. The filtrate was concentrated under reduced pressure to give the deprotected azide. 1 M Me_3P in THF (0.5 mL, 0.53 mmol) was added to a stirred soln of the azide in MeOH–H_2O (15:1, 8 mL). The mixture was stirred at r.t. for 16 h. Then, the solvent was removed under reduced pressure. To the resulting crude amine in H_2O (1.0 mL) was added a soln of succinimido (4′-hydroxy-1,1′-biphenyl-4-yl)acetate (30.34 mg, 0.08 mmol) in MeCN (5.0 mL), while maintaining the pH between 8.0–9.0 with sat. aq $NaHCO_3$. The mixture was stirred at r.t. for 48 h. The volatiles were evaporated and the residue was reconstituted into H_2O and applied onto a reversed-phase silica gel column pre-equilibrated in H_2O. The material was eluted with a gradient of MeOH–H_2O (0:1 to 3:10) to afford **12** [yield: 26.2 mg (50%)] as a white fluffy solid after a final lyophilization from H_2O. The solvent system EtOAc–MeOH–H_2O–AcOH (10:3:3:1) was used to monitor the reaction.

^{1}H NMR (600 MHz, CD_3OD): δ = 7.49–7.38 (m, 4 H, ArH), 7.30 (d, *J* = 8.0 Hz, 2 H, ArH), 7.03 (d, *J* = 8.0 Hz, 2 H, ArH), 4.38–4.29 (m, 3 H, propargylic CH_2, H_{1a}), 4.03 (s, 2 H, glycolyl CH_2CO), 3.95–3.85 (m, 3 H, H_{4a}, $H_{6a'}$, H_{8b}), 3.85–3.63 (m, 6 H, H_{2a}, H_{5a}, $H_{6a''}$, H_{4b}, H_{5b}, H_{6b}), 3.56 (s, 2 H, acetamido CH_2), 3.50–3.44 (m, 3 H, H_{3a}, H_{7b}, $H_{9b'}$), 3.20 (dd, *J* = 8.0, 12.0 Hz, 1 H, $H_{9b''}$), 2.83 (m, 2 H, $H_{3b\text{-}eq}$, alkynyl H), 1.61 (t, *J* = 12.0 Hz, 1 H, $H_{3b\text{-}ax}$).

HRMS: *m/z* [M – H]$^-$ calcd for $C_{34}H_{41}N_2O_{16}$: 733.2456; found: 733.2457.

Compound 13

To a soln of scaffold **8** (6.8 mg, 5.3 μmol) and sialoside **12** (12 mg, 16 μmol) in *t*-BuOH (0.5 mL) and H_2O (0.5 mL) were added $CuSO_4·5H_2O$ (1.9 mg) and sodium ascorbate (4.55 mg). The reaction mixture was stirred at r.t. for 48 h. The solvent was then lyophilized, and the solid residue was reconstituted into H_2O and purified using a Sephadex® column then a reversed-phase silica gel column. The material was eluted with a gradient of MeOH–H_2O (0:1 to 4:10) to afford cluster **13** [yield: 9 mg (48.5%)] as an orange fluffy solid after a final lyophilization from H_2O.

^{1}H NMR (600 MHz, D_2O + CD_3OD): δ = 7.99 (br s, 3 H, 3 triazole ring), 7.89 (d, *J* = 9.6 Hz, 3 H, H of nitrophenol), 7.41–7.36 (m, 15 H, 3 H of nitrophenol, 12 H of biphenyl), 7.27 (m, 6 H, H of biphenyl), 6.96 (br d, 3 H, H of nitrophenol), 6.84 (m, 8 H, 6 H of biphenyl, 2 H of gallic residue), 4.51 (m, 6 H, glycosidic CH_2), 4.35 (br d, 3 H, 3 H_{1a}), 4.35–3.53 (m, 96 H), 3.44–3.41 (m, 3 H, 3 $H_{9b'}$), 3.20 (m, 3 H, 3 $H_{9b''}$), 2.80 (br d, 3 H, 3 $H_{3b\text{-}eq}$), 1.67 (br t, 3 H, 3 $H_{3b\text{-}ax}$).

SDC of ERMS: *m/z* [M – H]$^{3-}$ calcd for $C_{155}H_{189}N_{22}O_{70}$: 1159.4; found: 1159.6.

Mass Spectrometric Analysis of Compound 13

Compound **13** was analyzed using a quadrupole ion trap mass spectrometer (QIT-MS) coupled with an electrospray interface (Bruker Esquire 3000 plus). The sample was dissolved in MeOH (Wako) and introduced into the ion source by infusion (flow rate: 120 μL/min). The parameters for the MS analysis were: (1) ion polarity, negative; (2) 'dry temperature': 250 °C; (3) nebulizer gas (N_2), 10 psi; (4) dry gas (N_2), 4.0 L/min; (5) 'Smart frag', off; (6) scan range, 500–1500 (MS/MS, 500–3000); (7) compound stability, 300%; (8) ICC target, 10000; (9) max acquisition time, 200 ms; (10) average; it adds to the above-mentioned in MS/MS; (11) target mass, 1159.6; (12) isolation width, 6.0; (13) cut off, 27.6% of the corresponding precursor ion; (14) fragmentation ampl., 1.3 V.

Supporting Information for this article is available online at http://www.thieme-connect.com/ejournals/toc/synthesis. Included are the ^{1}H NMR and mass spectra of compound **13**.

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