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Synthesis of bifunctional cationic compound for gene delivery

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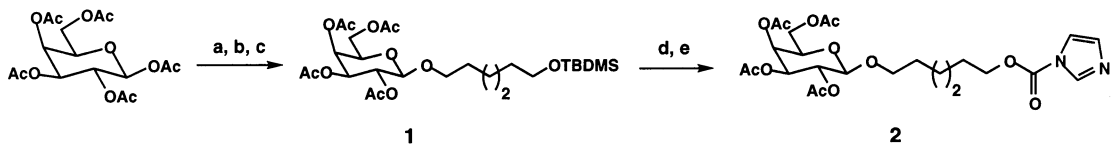
Abstract—Bifunctional cationic compound carrying trivalent galactosides as the cell targeting ligand and DAB-dendr-$(NH_2)_8$ (generation 2.0) as the DNA binding domain was synthesized for gene delivery to hepatocytes. DAB-dendr-$(NH_2)_4$ (generation 1.0) conjugated with a hydrocarbon chain was used as a scaffold for the attachment of three galactosides, while the other hydrocarbon end was linked with DAB-dendr-$(NH_2)_8$ (generation 2.0) through a carbamate bond. This design provided an effective entry for the synthesis of a polyamine compound having the cell targeting galactosyl ligand. Preliminary in vitro transfection results demonstrated that the bifunctional cationic compound could effectively deliver the gene into HepG2 cells.

The biological process where hepatic asialoglycoprotein receptor can recognize and uptake β-D-galactoside terminated glycoproteins is well known.[1] Previous efforts in understanding this recognition event through the probes of artificial glycopeptides having galactoside residues revealed that an enormous affinity enhancement was achieved by multivalent binding.[2] Recently, the use of asialoglycoprotein receptor as a target formed a basis for targeted gene delivery into hepatocytes.[3] As part of our program dealing with cationic compound-mediated gene delivery and cell targeting,[4] here we combined the clustered galactosyl ligand and poly(propylene imine) dendrimer onto a hydrocarbon chain. We expected that this bifunctional cationic compound could serve as a targetable DNA-vector which could efficiently deliver the gene into hepatocytes through an asialoglycoprotein receptor. The rationale for our design is that the DAB-dendr-$(NH_2)_8$ should spontaneously compact the DNA via an electrostatic interaction, the galactose residues on the other end of the molecule serve as the targeting ligand for hepatocytes, the hydrophobic chain in the middle provides a sheath around the DNA that protects it against degradation by the biological fluids.

β-D-galactose was converted to its hexyl spacer-armed derivative containing an activated imidazole carboxylic ester as the reactive end group (Scheme 1). The synthesis was started by chemoselective removal of anomeric acetyl group in pentaacetate β-D-galactose by hydrazine acetate.[5] Subsequent treatment of the resulting hemiacetal with large excess amounts of trichloroacetonitrile[6] in the presence of a catalytic amount of DBU[7] for 1.5 h at 0°C, gave the thermodynamically more stable α-trichloroacetimidate in an excellent yield. AgOTf promoted *O*-glycosylation gave the corresponding β-glycoside derivative **1** in 97% isolated yield.[8] The stereochemistry of compound **1** was confirmed by its ^1H NMR spectrum with the anomeric proton exhibiting a doublet with a coupling constant of 7.9 Hz centered at 4.44 ppm. Desilylation of **1** with tetrabutylammonium fluoride by a standard method released the primary



Scheme 1. *Reagents and conditions:* (a) NH_2NH_2–AcOH (1.1 equiv.), DMF, 60°C, 30 min, 100%; (b) CCl_3CN–CH_2Cl_2 (1:4 = v/v), cat. DBU, 90%; (c) 4 Å MS, $HO(CH_2)_6OTBDMS$ (0.5 equiv.), then AgOTf (0.3 equiv.), 0°C, 2 h, 97%; (d) Bu_4NF (1.5 equiv.), THF, rt, 12 h, 90%; (e) CDI (1.25 equiv.), DMAP (0.2 equiv.), CH_2Cl_2, rt, 1 h, 95%. DBU = 1,8-diazabicyclo[5,4,0]undec-7-ene, CDI = 1,1′-carbonyldiimidazole, DMAP = 4-(dimethylamino)pyridine.

Keywords: galactoside; polyamine dendrimer; targeting ligand; carbamate; 1,1′-carbonyldiimidazole; gene delivery.
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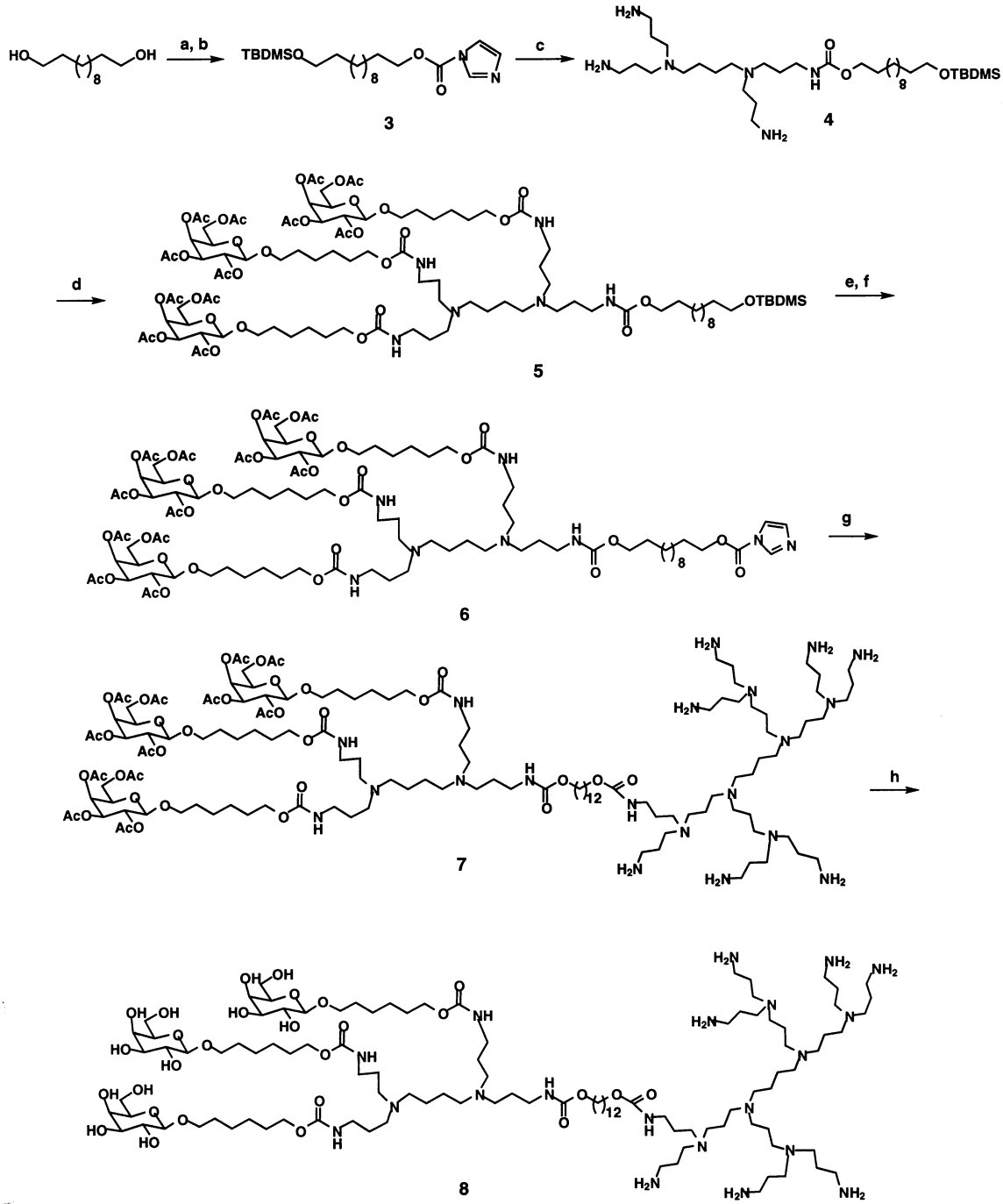
alcohol, which was activated with 1,1′-carbonyldiimidazole (CDI)[9] under DMAP[10] to afford the imidazole carboxylic ester **2**.[11]

Commercially available 1,12-diol was chosen as a hydrophobic linker, since it has two hydroxyl functionalities available for generating two carbamate bonds, which could bridge the cell surface targeting ligand—galactoside and the DNA binding domain—polyamine (Scheme 2). Treatment of 1,12-diol with 0.5 equiv. of TBDMSCl in the presence of imidazole afforded the corresponding mono-TBDMS derivative. The primary alcohol in the mono-TBDMS derivative was activated with CDI in the presence of 4-(dimethylamino)pyridine (DMAP) to give the imidazolide **3** in an excellent yield. Compound **3** was treated with excess amounts of DAB-dendr-$(NH_2)_4$ to give carbamate **4**, the excess amounts of unreacted DAB-dendr-$(NH_2)_4$ were removed by



Scheme 2. *Reagents and conditions:* (a) TBDMSCl (0.5 equiv.), imidazole (1 equiv.), DMF, 78%; (b) CDI (1.25 equiv.), DMAP (0.2 equiv.), CH_2Cl_2, rt, 1 h. 90%; (c) DAB-dendr-$(NH_2)_4$ (4 equiv.), dry MeCN, DMAP (0.2 equiv.), rt, 1 h. 85%; (d) Compound **2** (3.5 equiv.), THF, DMAP, reflux, 6 h, 50%; (e) Bu_4NF (1.5 equiv.), THF, rt, 18 h, 85%; (f) CDI (1.5 equiv.), DMAP (0.2 equiv.), CH_2Cl_2, rt, 2 h, 88%; (g) DAB-dendr-$(NH_2)_8$ (4 equiv.), THF, reflux, 1.5 h; (h) 0.04 M NaOMe–MeOH, rt, 2 h; then Dowex 50WX4-100. ca. 70% for two steps.

workup with 5% potassium hydroxide aqueous solution. At this stage, we used DAB-dendr-$(NH_2)_4$ (generation 1.0) as a potential scaffold to conjugate with spaced galactoside and expected that the primary amine in compound **4** could react with the terminal activated galactoside **2** in an S_N2 manner. Indeed, when compound **4** was reacted with an excess amount of imidazolide **2** in refluxing tetrahydrofuran (THF) for 6 h, the clustered trisaccharide derivative **5** was obtained in 50% separated yield.[12] Exposure of clustered trisaccharide silyl ether **5** to tetrabutylammonium floride in THF slowly liberated the primary alcohol, which was further activated with CDI to give the imidazole carboxylic ester **6**.[13] Treatment of **6** with excess amounts of DAB-dendr-$(NH_2)_8$ (generation 2.0) furnished the construction of the final carbamate bond. Removal of excess amounts of unreacted DAB-dendr-$(NH_2)_8$ by careful workup with 5% potassium hydroxide aqueous solution[14] led to the compound **7**.[15] Deprotection of peracetate in **7** was achieved under Zemplén conditions. After neutralization with acidic resin and concentration, the reaction residues were dialyzed against water in a membrane with molecular weight cut-off of 1000. Finally, lyophilization of the dialyzed compound afforded the desired product **8** in ca. 70% yield for the last two steps.[16]

Transfection efficiency of **8** was initially tested in HepG2 liver cells. Five nmol of compound **8** mixed with 1 μg of pCMV-Luc plasmid DNA was used for the transfection of HepG2 liver cells (5×10^4 cells per well in a 48-well plate). Using a standard transfection assay with luciferase as the reporter,[17] we harvested 64 ng of luciferase protein per mg of extracted proteins. These results demonstrated that the bifunctional cationic compound **8** could effectively deliver a gene into HepG2 cells.

In summary, we have developed an efficient route toward the synthesis of bifunctional cationic compound **8**. DAB-dendr-$(NH_2)_4$ (generation 1.0) conjugated with a hydrocarbon chain was used as a scaffold for the attachment of three galactosides, while the other hydrocarbon end was linked with DAB-dendr-$(NH_2)_8$ (generation 2.0) through a carbamate bond. This design provided an effective entry for the synthesis of a polyamine compound having the cell targeting galactosyl ligand. Preliminary in vitro transfection results demonstrated that the bifunctional cationic compound could effectively deliver a gene into HepG2 cells. Application of this compound for delivering a gene into the targeted cells in vivo is now underway.

Acknowledgements

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11. Compound **2**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.14 (s, 1H, imidazole ring), 7.44 (s, 1H, imidazole ring), 7.08 (s, 1H, imidazole ring), 5.39 (d, $J=3.2$ Hz, 1H, H-4), 5.20 (dd, $J=10.4$, 8 Hz, 1H, H-2), 5.01 (dd, $J=10.4$, 3.4 Hz, 2H, H-3), 4.45 (d, $J=8$ Hz, 1H, H-1), 4.41 (t, $J=6.6$ Hz, 2H, CH_2OCO), 4.20 (dd, $J=11.2$, 4.7 Hz, 1H, H-6a), 4.12 (dd, $J=11.6$, 6.9 Hz, 1H, H-6b), 3.90 (m, 2H, H-5 and alkyl chain H-1′), 3.48 (m, 1H, alkyl chain H-1′), 2.15 (s, 3H, Ac), 2.05 (s, 6H, 2×Ac), 1.99 (s, 3H, Ac), 1.78 (m, 2H, CH_2), 1.60 (m, 2H, CH_2), 1.42 (m, 4H, 2×CH_2) ppm.
12. Compound **5**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.51 (br, 4H, 4×$NHCO_2$), 5.36 (d, $J=3$ Hz, 3H, H-4), 5.19 (dd, $J=10.4$, 7.9 Hz, 3H, H-2), 4.99 (dd, $J=10.5$, 3.3 Hz, 3H, H-3), 4.42 (d, $J=7.8$ Hz, 3H, H-1), 4.10 (dd, $J=11.5$, 7 Hz, 3H, H-6a), 4.07 (dd, $J=11.2$, 6.9 Hz, 3H, H-6b), 3.99 (t, $J=6.5$ Hz, 8H, 4×CH_2OCO), 3.88 (m, 6H, H-5 and alkyl chain H-1′), 3.56 (t, $J=6.6$ Hz, 2H, CH_2OTBDMS), 3.44 (m, 3H, alkyl chain H-1′), 3.17 (brm, 8H, 4×CH_2NHCO_2), 2.50–2.40 (m, 12H, 2×$N(CH_2)_3$), 2.12 (s, 9H, 3×Ac), 2.02 (s, 18H, 6×Ac), 1.95 (s, 6H, 2×Ac), 1.70–1.50 (m, 24H, 12×CH_2), 1.40–1.20 (32H, 16×CH_2), 0.86 (s, 9H, t-Bu), 0.02 (s, 6H, 2×CH_3) ppm.
13. Compound **6**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 8.13 (s, 1H, imidazole ring), 7.43 (s, 1H, imidazole ring), 7.07 (s, 1H, imidazole ring), 5.48 (br, 4H, 4×$NHCO_2$), 5.39 (d, $J=3$ Hz, 3H, H-4), 5.19 (dd, $J=10.4$, 8 Hz, 3H, H-2), 4.98 (dd, $J=10.4$, 3.4 Hz, 3H, H-3), 4.45 (d, $J=8$ Hz, 3H, H-1), 4.41 (t, $J=6.6$ Hz, 2H, CH_2OCO), 4.18 (dd, $J=11.3$, 6.7 Hz, 3H, H-6a), 4.12 (dd, $J=11.3$, 7 Hz, 3H, H-6b), 4.02 (t, $J=6.6$ Hz, 8H, 4×CH_2OCO), 3.92 (m, 6H, H-5 and alkyl chain H-1′), 3.47 (m, 3H, alkyl chain H-1′), 3.20 (brm, 8H, 4×CH_2NHCO), 2.50–2.35(m, 12H, 2×$N(CH_2)_3$), 2.15 (s, 9H, 3×Ac), 2.07 (s, 18H, 6×Ac), 1.98 (s, 9H, 3×Ac), 1.70–1.50 (m, 24H, 12×CH_2), 1.50–1.20 (m, 32H, 16×CH_2) ppm.
14. When the reaction mixture diluted with 50 mL of chloroform and washed with saturated NaCl aqueous solution, a formation of emulsions occurred. However, when the organic layer was treated with 5% KOH aqueous solution combined with 10 mL of methanol, a better phase separation was obtained.
15. Compound **7**: ^{1}H NMR (300 MHz, $CDCl_3$) δ 5.55 (br, 5H, 5×$NHCO_2$), 5.33 (d, $J=3$ Hz, 3H, H-4), 5.14 (dd, $J=10.4$, 8 Hz, 3H, H-2), 4.96 (dd, $J=10.5$, 3.3 Hz, 3H, H-3), 4.40 (d, $J=7.9$ Hz, 3H, H-1), 4.10 (dd, $J=11.1$, 6.7 Hz, 3H, H-6a), 4.08 (dd, $J=11.1$, 6.6 Hz, 3H, H-6b), 3.96 (t, $J=6.6$ Hz, 10H, 5×CH_2OCO), 3.85 (m, 6H, H-5 and alkyl chain H-1′), 3.40 (m, 3H, alkyl chain H-1′), 3.20 (m, 10H, 5×CH_2NHCO), 2.66 (t, $J=7$ Hz, 14H, 7×CH_2NH_2), 2.45–2.22 (m, 48H, 8×$N(CH_2)_3$), 2.09 (s, 9H, 3×Ac), 2.00 (s, 18H, 6×Ac), 1.93 (s, 9H, 3×Ac), 1.70–1.20 (m, 98H, 7×NH_2 and 42×CH_2) ppm.
16. Compound **8**: ^{1}H NMR (300 MHz, CD_3OD) δ 4.17 (d, $J=6.9$ Hz, 3H, H-1), 3.98 (m, 10H, 5×CH_2OCO), 3.90–3.80 (m, 6H, alkyl chain H-1′ and H-4), 3.70 (m, 6H, H-6a and H-6b), 3.54–3.45 (m, 12H, H-2, H-3, H-5 and alkyl chain H-1′), 3.15 (t, $J=6.6$ Hz, 10H, 5×CH_2NHCO_2), 2.75 (m. 14H, 7×CH_2NH_2), 2.45 (m, 48H, 8×$N(CH_2)_3$), 170–1.20 (m, 84H, 42×CH_2) ppm.
17. For detailed experimental procedure of in vitro transfection see: reference 4b.