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Chemical and enzymatic synthesis of neoglycolipids in the presence of cyclodextrins

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Abstract

The efficiency of cyclodextrins (CDs), α-CD, β-CD and γ-CD, for the blotting of hydrophobic substrates to water-soluble polymers and for the synthesis of neoglycolipids using glycosyltransferase is described. CDs did not disturb HPLC purification and did not bring lather. These merits are superior to surfactants commonly used in such a case. The utility of CDs as a novel and efficient supporting material for enzymatic glycosylation is also discussed.

Keywords: Enzyme; Glycosyltransferase; Cyclodextrin; Polymer-supported synthesis; Carbohydrate

The development of carbohydrate analysis has revealed the biological importance of oligosaccharides,[1–3] and carbohydrate chemistry is expected to contribute to the pharmaceutical and industrial sectors. From this point of view, we have developed new methods to prepare oligosaccharides easily using glycosyltransferases, which can control glycosylation with precise regio- and stereoselectivities. As a part of our researches, we have reported the parallel synthesis of glycopeptides in μmol scale to produce an oligopeptide-focused library by the molecular transporter between solid-phase and water-soluble polymers.[4,5] Our next topics of interest have been focused on preparing glycolipid derivatives. Although we have reported the utility of the water-soluble polymer in enzymatic reactions, the key point should still be the solubility of neoglycolipids, which are generally more hydrophobic than glycopeptides.

To increase the solubility of substrates in enzymatic reactions, it is common to use a small amount of a polar solvent, like DMSO, or to use surfactants like cholic acid sodium salt and sodium dodecyl sulfate (SDS) as ionic surfactants and octylphenyl ethoxylates (Triton-X) as a non-ionic surfactant. Although surfactants are helpful for enzymatic reactions, only a limited concentration of substrates becomes soluble and using a large quantity of surfactants resulted in other problems during purification stages.

In this Letter, we studied the utility of simple cyclodextrins (CDs) for the synthesis of neoglycolipids using glycosyltransferase. CDs have the ability to include the hydrophobic area of a compound, and the formed complex becomes soluble in water due to hydroxyl groups orientated on the surface of CDs. Thus, we thought CDs would have similar properties with surfactants in enzymatic reactions. While there are reports of the use of CDs for the enzymatic glycosylations,[6–8] there are no systematic studies of the effect for the enzymatic synthesis of neoglycolipids, which have large hydrophobic parts; namely the relationship between concentrations of CD and substrates; or the

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differences between α-, β- and γ-CD. Thus, here, we studied the details of the efficiency of cyclodextrins for galactosyltransferase in vitro to synthesize neoglycolipids in a large scale.

Until now, there have been many reports of glycosylation studies, namely chemical coupling, enzymatic syntheses in vitro, and syntheses using cell function in vivo. The combination of both chemical and enzymatic methods has become a common strategy nowadays and has been called chemoenzymatic synthesis. As shown in Figure 1, we have attempted to also add cell-media synthesis, which we named cellular-chemoenzymatic synthesis, as a triple-factor combination method, to make oligosaccharide synthesis easier.

Yamagata, Sato, Hatanaka et al. have reported 12-azidododecyl lactose as a primer, a starting material for the synthesis of GM3 analogue by cells.[9–11] We thought if we could conjugate it with the reported water-soluble polymer[4,5] through a suitable linker, we could modify more complicated oligosaccharides by glycosyltransferase in vitro.

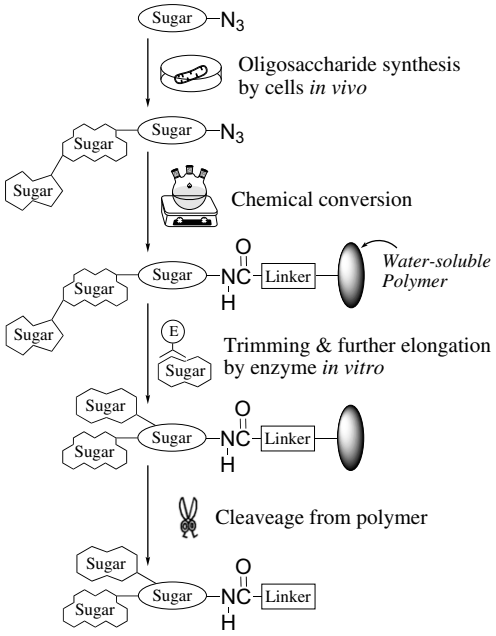
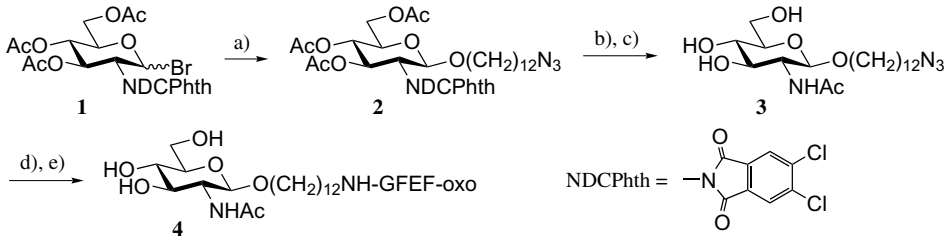


Fig. 1. Scheme of the triple-factor combination method for oligosaccharide synthesis.

Based on this strategy, the linker should be designed to have the following: (i) a functional group to couple with the azido group onto oligosaccharides synthesized by cells; (ii) a functional group to conjugate with the water-soluble polymer; (iii) a part to be released from the polymer; and (iv) leaving a UV tag on the synthesized oligosaccharides was favourable to apply further researches, like the development of glycan array.[11] According to (i), the carboxyl group would be suitable for application in the Staudinger reaction.[12–14] In the case of amino acids' carboxyl groups, glycine should be used because conjugation with azido would lead to racemization at α-position. According to (ii), conjugation with ketone and aminooxy groups onto the linker and the water-soluble polymer, respectively, has already been reported.[4,5] According to (iii), the reaction to release synthesized oligosaccharides from the polymer is preferred under mild conditions because the target oligosaccharides have multi-functional groups. Thus, our first option was an enzymatic reaction. BLase protease[15] can hydrolyze a bond between amino acids of Glu and Phe, and hydrophobic amino acids situated next to Glu could accelerate the reaction. In fact, we already showed the utility of the BLase as a releasing reagent from the polymer.[5] In summary, we designed the linker as $CH_3C(=O)CH_2CH_2CH_2C(=O)$-Phe-Glu-Phe-Gly-OH (oxo-FEFG),[16] which would result in leaving a UV tag, phenylalanine, on the side of the synthesized neoglycolipid when released from the polymer.

To test along the above strategy, we chose a glucosamine derivative as a model material. 12-azidododecyl 2-acetoamido-2-deoxy-β-D-glucopyranoside (**3**)[17] was synthesized from bromide (**1**)[18] in 45% yield in three steps. Instead of applying the Staudinger reaction, in this case, reduction with triphenylphosphine followed by coupling with the peptide linker, oxo-FEFG,[16] and deprotection gave **4**,[17] which the linker was successfully introduced to the azidoalkyl group (Scheme 1).



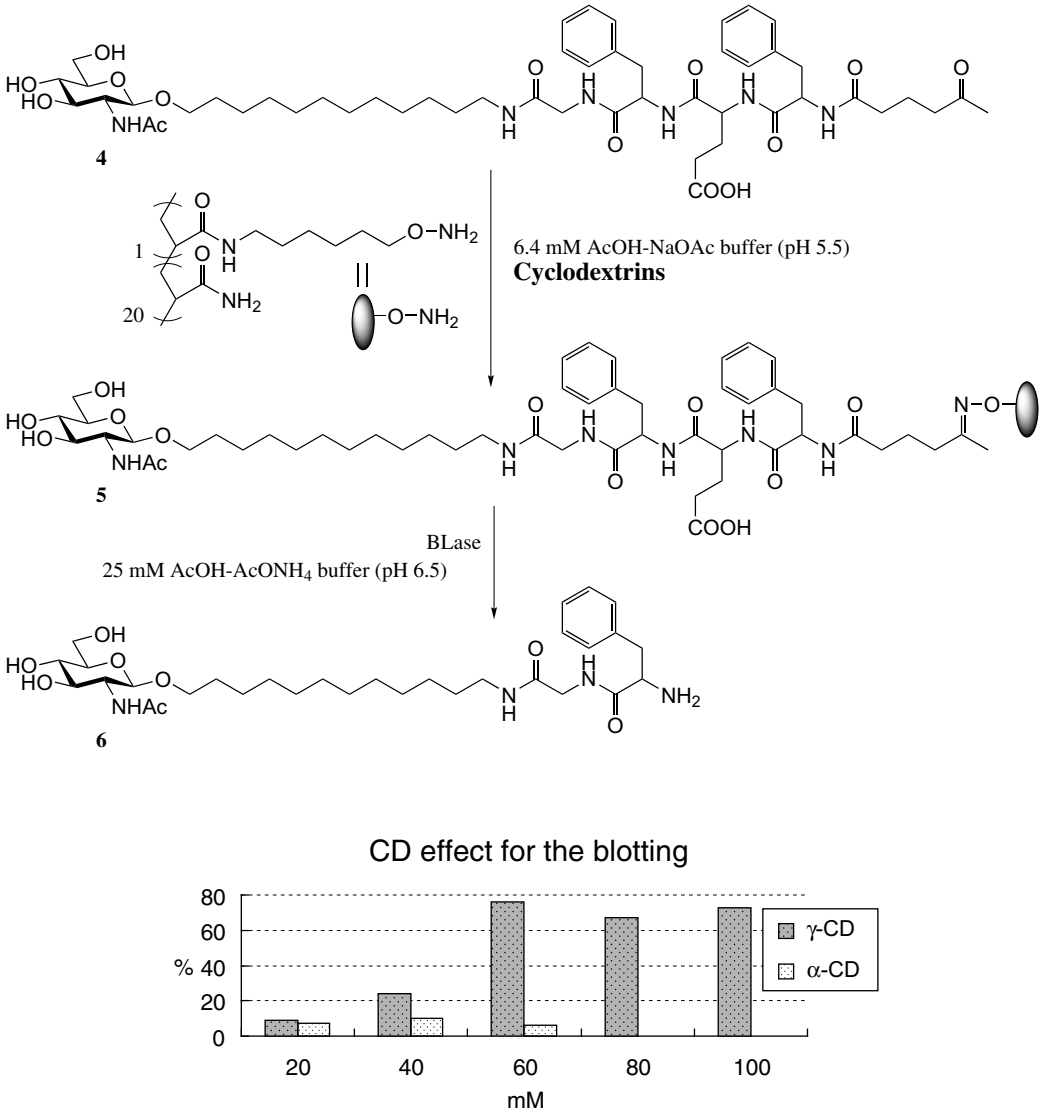
Scheme 1. Preparation of model primer, *N*-acetyl-glucosamine derivative and chemical modification. Reagents and conditions: (a) $N_3(CH_2)_{12}OH$ (1.0 equiv and 1.4 equiv of **1** was used), AgOTf (1.4 equiv), *s*-collidine (1.0 equiv), CH_2Cl_2, (3 ml/1g of **1**), −20 °C, 2 h (59% from $N_3(CH_2)_{12}OH$); (b)[18] $H_2NCH_2CH_2NH_2$ (1 ml/1 g of **2**),[17] MeOH (3 ml/1 g of **2**), rt, 18 h, then Ac_2O (2 ml/1 g of **2**), Py (8 ml/1 g of **2**), rt, 5 h (91%): (c) NaOMe (cat.), MeOH, rt, 1 h (97%); (d) PPh_3 (1.0 equiv), EtOH (10 ml/1 g of **3**), 50 °C, 18 h (90%); (e) oxo-FE(OBn)FG[16] (1.1 equiv), WSC (1.2 equiv), HOBt (1.2 equiv), Et_3N (1.0 equiv), DMF (5 ml/100 mg of **3**), THF (5 ml/100 mg of **3**), rt, 1 h, then Pd–C (cat.), dioxane (1.5 ml/100 mg of **3**), water (1.5 ml/100 mg of **3**), 50 °C, 1 h, (87%).

Next, we attempted to introduce **4** onto the water-soluble polymer, but we only obtained 3% of **6**[17] in the simple steps of blotting to and cleavage from the polymer. We thought this low yield was due to the solubility of **4** in water buffer, and we found that γ-CD could improve the yield dramatically, blotting with 60 mM γ-CD in sodium acetate buffer and cleavage steps resulted in 76% yield (Scheme 2).

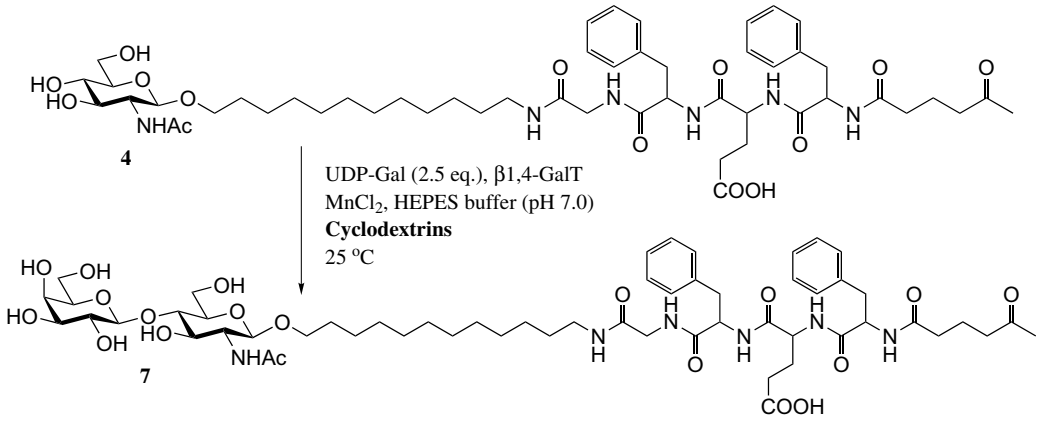
As we found an efficiency of γ-CD in this simple blotting stage, it would be natural to think that additional CDs for **4** without blotting would be enough for the reactions using galactosyltransferase (GalT). However, without any additional reagents, *N*-acetyl-lactosamine disaccharide (**7**) was not produced at all even in 0.1 mM concentration condition, suggesting that the CDs turned the impossible into possible.[19]

Three types of CD are very common, α-CD, β-CD and γ-CD, which are composed of six, seven and eight glucose units, respectively. Although all glucose units have α1-4 linkage, only β-CD's solubility is low. Therefore, we have mixed only 10 mM of β-CD for the study although α-CD and γ-CD were tested up to 100 mM (Scheme 3).

Table 1 shows the glycosylation speeds of the beginning of the reactions (V_0, mM/h) and are marked if the reactions were completed. Although α-CD was not as effective even at high concentrations of α-CD (100 mM) against low concentrations of **4** (2 mM), 10 mM β-CD worked well for the glycosylation until 4 mM of **4**, but unfortunately we could not increase the concentration of β-CD further due to its solubility. Regarding γ-CD, 30 mM, 50 mM, 70 mM and 90 mM γ-CD could lead to **7** quantitatively by the conditions of 2 mM, 4 mM, 6 mM and even 8 mM of **4**, respectively. We would like to note that the reaction mixtures of control and 10 mM γ-CD became viscous, and 90 mM led to a white suspension although the reaction continued to proceed; 30–70 mM resulted in clear solutions (Fig. 2).



Scheme 2. Preliminary experiment for a model primer blotting to and releasing from the water-soluble polymer. Cyclodextrins, which were added in the blotting stage only, improved the results dramatically.



Scheme 3. Study of cyclodextrins' effect for the synthesis of *N*-acetyl-lactosamine derivative (**7**) by β1,4-galactosyltransferase.

Table 1
Reaction speed (V_0, mM/h) by galactosyltransferase from **4** to **7**, calculated from the reaction ratio at 1 h, 2 h and 3 h[18]

		Concentration of **4**				
		2 mM	4 mM	6 mM	8 mM	10 mM
α-CD	100 mM	0.02	NR			
β-CD	10 mM	0.14[c]	0.32[b]	0.06	NR	
γ-CD	10 mM	NR				
	20 mM	0.03	NR			
	30 mM	0.28[a]	0.03	NR		
	50 mM	0.22[a]	0.20[c]	0.05	NR	
	70 mM	0.26[a]	0.32[b]	0.24[b]	0.06	NR
	90 mM	0.26[a]	0.28[b]	0.24[b]	0.36[b]	0.07
Triton X-100 1%		0.16[b]	0.20	0.36		

NR means no reaction. Blank areas indicate reactions that were not performed.
[a, b, c] Indicate that the reactions were completed within 12 h, 24 h and 48 h, respectively.

In contrast, the reaction mixture with 1% of Triton X-100 became a gel phase (photo not shown), so the reaction was not concluded in either case of 4 mM and 6 mM substrates even though the V_0 were similar to those with CDs.

In conclusion, we have demonstrated the efficiency of cyclodextrins for the enzymatic synthesis of neoglycolipids that are barely water soluble, and based on this study we could make a bridge between oligosaccharide syntheses using cell function in vivo and enzymatic synthesis in vitro and established cellular-chemoenzymatic synthesis.

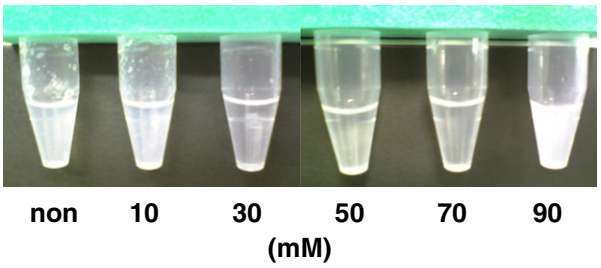


Fig. 2. Photos of reaction flasks for 2 mM substrates. Concentration of the present γ-CD is shown. The control and 10 mM γ-CD formed a gel and the 90 mM became a white suspension, while the 30 mM, 50 mM and 70 mM led to clear solutions.

Increasing the concentration of γ-CD led to a raise in the limitation of the concentration of substrate **4**, which suggested that CDs did not work as surfactants nor as phase transfer systems but as molecular recognition systems. Thus, although γ-CD worked well, α-CD was not as efficient even at high concentrations, because the number of recognition parts was different, namely γ-CD supposed to involve phenyl groups on the linker, while α-CD might not. In practicality, CDs did not prevent HPLC purification. CDs eluted completely in early fractions and we could not observe any CD with purified **7** by NMR, although we had planned to use α-amylase to convert γ-CD to glucose if the product was contaminated. Finally, regarding the scale of the reactions, γ-CD could allow for the synthesis of **7** in the 8 mM condition, that is, 8 mg of **4** in 1 ml buffer could be converted. This is a rather high concentration of neoglycolipid prepared by glycosyltransferase in vitro. In addition, modified CDs, like methylated CDs with higher solubility, are also available, which suggest the possibility to further increase the reaction scale.

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16. Oxo-FEFG was synthesized on Fmoc-Gly-OH preloaded with 2-chlorotrityl chloride resin (l-glycine-2-chlorotrityl resin, 0.80 mmol/g), DIEA (840 μl, 4.8 mmol) was added to the mixture of the resin (1 g) pre-washed by CH_2Cl_2, Fmoc-Phe-OH (930 mg, 2.4 mmol), HBTU (910 mg, 2.4 mmol) and HOBt (320 mg, 2.4 mmol) in DMF (5 ml), and the mixture was stirred for 10 min under occasional microwave irradiation at 50 °C. This coupling process was performed twice. After washing the resin with CH_2Cl_2 and DMF, 20% piperidine in DMF (4 ml) was added and the mixture was stirred for 5 min under occasional microwave irradiation at 50 °C. After washing the resin with CH_2Cl_2 and DMF, the same coupling procedure was carried out using Fmoc-Glu(OBn)-OH (1.1 g, 2.4 mmol). We should note here that the deprotection of the Fmoc group by piperidine was performed for 2 min at rt instead of for 10 min under microwave irradiation to prevent byproducts that would cause lactamization of the glutamic acid. After the introduction of the Glu moiety as above, Fmoc-Phe-OH was conjugated by the same procedure for 2 min at rt for the deprotection of Fmoc, and finally 5-oxohexanoic acid (290 μl, 2.4 mmol) was used in the same procedure as above for the introduction of a ketone group. Treatment of the resin washed with CH_2Cl_2 and DMF in 10% TFA in dichloromethane (20 ml) or AcOH/CF_3CH_2OH/CH_2Cl_2 (2:2:6, 20 ml) for 1 h at rt followed by HPLC purification [column: Inertsil ODS-3, ∅ 20 mm × 250 mm; eluate: acetonitrile, gradient increase from 20% to 90% over 50 min; column oven temperature: 40 °C; flow rate: 5 ml/min] gave $CH_3C(=O)$-$CH_2CH_2CH_2C(=O)$–FE(OBn)FG in 57% and 58% yields, respectively. ^{1}H NMR (400 MHz, $(CD_3)_2S=O$): δ 8.34 (1H, t, $J = 5.8$ Hz, Gly-N*H*), 8.03 (1H, d, $J = 7.7$ Hz, Glu-N*H*), 8.01 (1H, d, $J = 8.2$ Hz, Phe-N*H*), 7.96 (1H, d, $J = 8.3$ Hz, Phe-N*H*), 5.09 (2H, s, $PhCH_2O$–), 4.55 (1H, m, Phe-Hα), 4.52 (1H, m, Phe-Hα), 4.25 (1H, m, Glu-Hα), 3.80 (1H, dd, $J = 17.6$, 5.8 Hz, Gly-Hα), 3.75 (1H, dd, $J = 17.6$, 5.8 Hz, Gly-Hα), 3.05 (1H, dd, $J = 13.9$, 4.4 Hz, Phe-Hβ), 2.94 (1H, dd, $J = 13.9$, 3.9 Hz, Phe-Hβ), 2.80 (1H, dd, $J = 13.9$, 9.5 Hz, Phe-Hβ), 2.68 (1H, dd, $J = 13.9$, 10.7 Hz, Phe-Hβ), 2.29 (2H, m, Glu-Hγ), 2.18 (2H, t, $J = 7.3$, $–CH_2CH_2C(=O)$–), 1.98 (2H, t, $J = 7.3$, $–CH_2CH_2C(=O)$–), 1.97 (3H, s, $CH_3C(=O)$–), 1.89 (1H, m, Glu-Hβ), 1.76 (1H, m, Glu-Hβ), 1.52 (2H, qui, $J = 7.3$, $–C(=O)CH_2CH_2CH_2$-$C(=O)$–). ^{13}C NMR (100 MHz, $(CD_3)_2S=O$): δ 207.97, 172.20, 171.72, 171.45, 171.16, 170.99, 170.51, 138.04, 137.56, 136.18, 129.14, 129.08, 128.39, 127.96, 127.95, 127.93, 127.88, 126.19, 126.11, 65.42, 53.59, 53.51, 51.80, 41.65, 40.62, 37.51, 37.17, 34.12, 29.86, 29.65, 27.34, 19.22. MALDI-TOF-MASS *m*/*z* for $C_{38}H_{44}N_4O_9$ $[M+Na]^+$ calcd 723.30, found 723.58; $[M+K]^+$ calcd 739.27, found 739.60. ESI HR MS *m*/*z* for $C_{38}H_{44}N_4O_9$ $[M+H^+]$ calcd 701.31865, found 701.32016.
17. Characterization of compounds. Compound **2**: ^{1}H NMR (400 MHz, $CDCl_3$): δ 7.96 (2H, s, aromatic-*H*), 5.74 (1H, dd, $J = 10.7$, 9.2 Hz, H-3), 5.34 (1H, d, $J = 8.5$ Hz, H-1), 5.19 (1H, dd, $J = 9.9$, 9.2 Hz, H-4), 4.34 (1H, dd, $J = 12.3$, 4.6 Hz, H-6), 4.29 (1H, dd, $J = 10.7$, 8.5 Hz, H-2), 4.19 (1H, dd, $J = 12.3$, 2.3 Hz, H-6), 3.28 (2H, t, $J = 7.0$ Hz, N_3CH_2–), 2.13 (3H, s, $CH_3C(=O)$–), 2.06 (3H, s, $CH_3C(=O)$–), 1.90 (3H, s, $CH_3C(=O)$–). ^{13}C NMR (100 MHz, $CDCl_3$): δ 170.72, 170.32, 169.43, 139.39, 130.52, 125.69, 97.97, 77.23, 71.89, 70.84, 70.21, 68.90, 62.05, 55.15, 51.51, 29.59, 29.52, 29.48, 29.45, 29.21, 29.16, 28.85, 26.72, 25.87, 20.77, 20.63, 20.47. ESI HR MS *m*/*z* for $C_{32}H_{42}Cl_2N_4O_{10}$ $[M+Na^+]$ calcd 735.21757, found 735.22036. **3**: ^{1}H NMR (400 MHz, MeOD): δ 4.41 (1H, d, $J = 8.4$ Hz, H-1), 3.70 (1H, dd, $J = 11.6$, 5.6 Hz, H-6), 3.64 (1H, dd, $J = 10.3$, 8.4 Hz, H-2), 3.29 (2H, t, $J = 6.7$ Hz, N_3CH_2–), 1.99 (3H, s, $CH_3C(=O)$–). ^{13}C NMR (100 MHz, MeOD): δ 173.63, 102.75, 77.97, 76.11, 72.22, 70.62, 62.87, 57.49, 52.49, 30.82, 30.73, 30.71, 30.70, 30.65, 30.56, 30.29, 29.94, 27.85, 27.17, 23.04. ESI HR MS *m*/*z* for $C_{20}H_{38}N_4O_6$ $[M+Na^+]$ calcd 453.26890, found 453.26837. Compound **4**: ^{1}H NMR (400 MHz, MeOD): δ 7.29–7.18 (10H, m, aromatic-*H*), 4.56 (1H, dd, $J = 9.9$, 5.0 Hz, Phe-Hα), 4.46 (1H, dd, $J = 9.2$, 6.0 Hz, Phe-Hα), 4.38 (1H, d, $J = 8.4$ Hz, H-1), 4.20 (1H, dd, $J = 8.3$, 5.3 Hz, Glu-Hα), 3.91 (1H, d, $J = 16.8$ Hz, Gly-Hα), 3.67 (1H, dd, $J = 11.9$, 5.6 Hz, H-6), 3.65 (1H, d, $J = 16.8$ Hz, Gly-Hα), 3.62 (1H, dd, $J = 10.3$, 8.4 Hz, H-2), 3.10 (1H, dd, $J = 13.9$, 5.0 Hz, Phe-Hβ), 3.01 (1H, dd, $J = 13.9$, 9.2 Hz, Phe-Hβ), 2.84 (1H, dd, $J = 13.9$, 9.9 Hz, Phe-Hβ), 2.33 (2H, t, $J = 7.3$, $–CH_2CH_2C(=O)$–), 2.06 (3H, s, $CH_3C(=O)$–), 1.96 (3H, s, $CH_3C(=O)$–), 1.72 (2H, qui, $J = 7.3$ Hz, $–C(=O)CH_2CH_2$-$CH_2C(=O)$–). ^{13}C NMR (100 MHz, MeOD): δ 211.11, 176.90, 176.01, 174.56, 173.90, 173.87, 173.67, 171.38, 138.50, 130.33, 130.30, 129.67, 129.58, 127.97, 127.91, 102.78, 78.02, 76.16, 72.27, 70.65, 62.91, 57.54, 56.97, 56.34, 54.86, 43.70, 43.11, 40.58, 38.46, 37.95, 35.72, 31.22, 30.85, 30.78, 30.77, 30.74, 30.58, 30.49, 30.43, 29.94, 28.00, 27.66, 27.19, 23.08, 20.75. ESI HR MS *m*/*z* for $C_{51}H_{76}N_6O_{14}$ $[M+Na^+]$ calcd 1019.53172, found 1019.53166. **6**: ^{1}H NMR (400 MHz, MeOD): δ 7.39–7.28 (5H, m, aromatic-*H*), 4.38 (1H, d, $J = 8.4$ Hz, H-1), 4.07 (1H, dd, $J = 8.0$, 6.0 Hz, Phe-Hα), 3.94 (1H, d, $J = 16.4$ Hz, Gly-Hα), 3.72 (1H, d, $J = 16.4$ Hz, Gly-Hα), 3.68 (1H, dd, $J = 11.9$, 5.6 Hz, H-6), 3.62 (1H, dd, $J = 10.3$, 8.4 Hz, H-2), 3.24 (1H, dd, $J = 14.0$, 6.5 Hz, Phe-Hβ), 3.18 (2H, t, $J = 7.1$, $–OCH_2–$ or $–NHCH_2–$), 3.04 (1H, dd, $J = 14.0$, 8.0 Hz, Phe-Hβ), 1.97 (3H, s, $CH_3C(=O)$–). ^{13}C NMR (100 MHz, MeOD): δ 173.64, 170.70, 173.07, 135.80, 130.50, 130.17, 128.91, 102.80, 78.00, 76.13, 72.24, 70.65, 62.90, 57.49, 56.01, 43.12, 40.58, 38.63, 30.80, 30.72, 30.70, 30.64, 30.54, 30.41, 27.99, 27.18, 23.04. ESI HR MS *m*/*z* for $C_{31}H_{52}N_4O_8$ $[M+H^+]$ calcd 609.38634, found 609.38566. Compound **7**: ^{1}H NMR (400 MHz, $(CD_3)_2S=O$): δ 8.25 (1H, t, $J = 5.7$ Hz, Gly-N*H*), 8.06 (1H, d, $J = 7.4$ Hz, Phe-N*H*), 8.02 (1H, d, $J = 8.4$ Hz, Phe-N*H*), 7.71 (1H, d, $J = 8.6$ Hz, Glu-N*H*), 7.59 (1H, t, $J = 5.5$ Hz, $–CH_2NHC(=O)$–), 7.26–7.14 (10H, m, aromatic-*H*), 5.05 (1H, br, –O*H*), 4.76 (1H, br, –O*H*), 4.61 (1H, br, –O*H*), 4.52 (1H, m, Phe-Hα), 4.45 (1H, m, Phe-Hα), 4.28 (1H, d, $J = 7.9$ Hz, H-1 or H-1′), 4.23 (1H, d, $J = 7.8$ Hz, H-1 or H-1′), 2.95 (1H, dd, $J = 13.9$, 3.8 Hz, Phe-Hβ), 2.84 (1H, dd, $J = 13.7$, 9.1 Hz, Phe-Hβ), 2.68 (1H, dd, $J = 13.9$, 10.7 Hz, Phe-Hβ), 2.19 (2H, t, $J = 7.3$, $–CH_2CH_2C(=O)$–), 1.99 (3H, s, $CH_3C(=O)$–), 1.53 (2H, qui, $J = 7.3$, $–C(=O)CH_2CH_2CH_2C(=O)$–). ^{13}C NMR (100 MHz, $(CD_3)_2S=O$): δ 208.34, 174.27, 172.14, 171.92, 171.48, 171.41, 168.95, 168.56, 137.41, 137.91, 129.46, 129.43, 128.39, 128.27, 126.62,104.35, 101.23, 81.83, 75.92, 75.35, 73.57, 72.64, 70.95, 68.78, 68.53, 60.83, 55.13, 54.56, 53.96, 34.49, 30.28, 30.03, 29.47, 29.38, 29.16, 29.11, 27.61, 26.71, 25.75, 23.30, 19.58. ESI HR MS *m*/*z* for $C_{57}H_{86}N_6O_{19}$ $[M+Na^+]$ calcd 1181.58454, found 1181.58306.
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19. Typical procedure of Scheme 3 for 2 mM **4** with 90 mM γ-CD: **4** (1.0 mg, 1.0 mol) was galactosylated using 25 mU of β1,4-GalT (SIGMA, G-5507, practically 1.7 mg was dissolved in 1.7 ml water as 3 U/ml, and used), 50 μl of 50 mM UDP-Gal and 300 μl of 150 mM γ-CD in a total volume of 500 μl of 50 mM HEPES buffer, pH 7.0, and 10 mM $MnCl_2$. Incubation was carried out at 25 °C and the reaction was monitored every hour with 5 μl sampling by HPLC [column: Inertsil ODS-3, ∅ 4.6 mm × 250 mm; eluate: acetonitrile, gradient increase from 30% to 40% over 30 min; column oven temperature: 30 °C; 220 nm UV detector; flow rate: 1 ml/min] $t_R = 24.5$ min for **7** and 27.5 min for **4**. In the case of the reaction for **4** (5.00 mg in total volume 2.5 ml, 2 mM) with 50 mM γ-CD, HPLC purification gave 5.78 mg of **7** (99%) [column: Inertsil ODS-3, ∅ 20 mm × 250 mm; eluate: 10% acetonitrile for 20 min followed by gradient increase from 10% to 30% over 10 min and 30% to 45% over 50 min; column oven temperature: 40 °C; 220 nm UV detector; flow rate: 5 ml/min. γ-CD was eluted out between 8 and 23 min, which was detected by MALDI-TOF MS, and **7** was collected from the fractions from 49 to 53 min].