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Cyclodextrin-assisted Glycan Chain Extension on a Protected Glycosyl Amino Acid

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Abstract—By the use of cyclodextrins, we have enhanced the solubility of the protected amino acid glycan Fmoc-Thr(GalNAcα1)-O*t*Bu (**1b**) up to 100-fold. This improvement enabled us to carry out an enzymatic glycosylation employing a β-galactosidase in combination with an α2,3-sialyltransferase without the aid of organic cosolvents. After optimization of the one-pot reaction, the sialylated core 1 structure Fmoc-Thr[Neu5Ac(α2-3)Gal(β1-3)GalNAcα1]-O*t*Bu (**3b**) could be obtained with 50% yield. © 2000 Elsevier Science Ltd. All rights reserved.

Introduction

Due to the important role of glycoproteins in cell recognition processes,[1,2] there is an increasing need for the synthesis of biologically active glycopeptides. They can be obtained either by enzymatic glycosylation of the synthetic peptides or by chemical synthesis using glycosylated amino acids as building blocks.[3] Enzymatic synthesis using glycosyltransferases,[4,5] the in vivo catalysts, are very efficient, but certain key glycosyltransferase activities are still not available in abundancy from recombinant systems. Alternatively, glycosidases, which catalyze hydrolysis in vivo, can be used for the formation of glycans in vitro. In order to improve poor yields resulting from product hydrolysis, combined use of glycosidases and transferases in multi-enzyme systems has been developed.[6,7] Recently, we reported the synthesis of an *O*-glycan core structure linked to a benzylic residue catalyzed by a galactosidase and a GlcNAc-transferase in a one-pot reaction with 90% yield.[8]

Enzymatic formation of glycosylated amino acid building blocks for solid-phase synthesis is difficult, because the protected amino acids are poorly soluble in aqueous systems. A non-protected threonin derivative of the sialyl-T containing building block **3b** was synthesized by Gambert and Thiem.[9] Organic cosolvents might be used for glycosylation of genuine building blocks, but in many cases they are not tolerated by the enzymes. Therefore, enzymatic glycosylations of protected amino acids gave yields of 15% or lower depending on the solubility of the substrate.[10] By a galactosidase-catalyzed transglycosylation with DMF as a cosolvent, Ajisaka and coworkers achieved a yield of 23% with regard to the amino acid substrate.[11]

Results and Discussion

The aim of this study was to combine enzymatic and chemical methods to efficiently obtain the sialyl-T containing building block **3b** from the glycosyl amino acid **1b**.

As the galactosyltransferase for the synthesis of the core 1 structure **2b** is only available from natural sources in small amounts,[12–14] the glycan chain of the protected *O*-glycosyl amino acid **1b**, which was obtained by chemical synthesis,[15,16] was extended using a commercially available galactosidase from bovine testes [E.C. 3.2.1.23] in combination with the core 1-specific human α2,3-sialyltransferase, ST3GalI [E.C. 2.4.99.4][17,18] (Fig. 1).

A soluble form of ST3GalI was expressed in Sf9 cells grown in serum-free medium and partially purified by consecutive chromatography on Amberlite IRA-95, DEAE- and SP-Sepharose as described previously.[19] In an alternative approach, purification by chromatography was avoided. Sialyltransferase containing medium was diafiltrated against MES buffer using an ultrafiltration membrane. Thus, a crude enzyme preparation was obtained.

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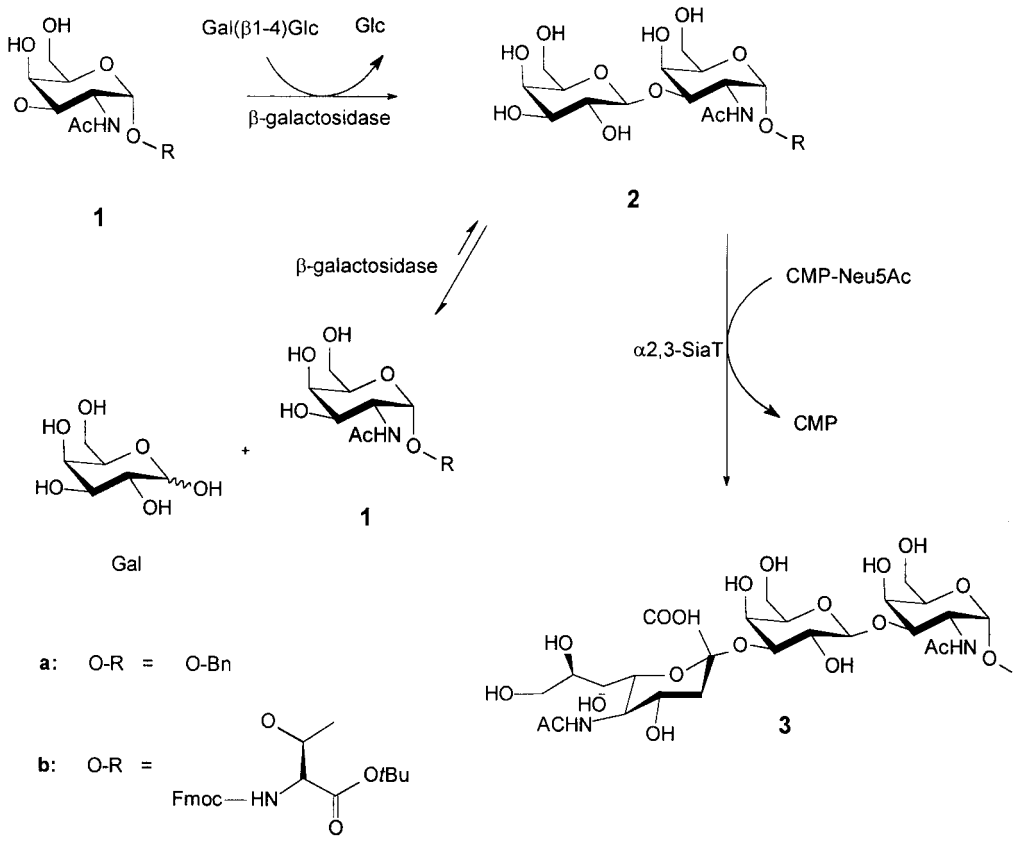

Figure 1. Simplified reaction scheme of the galactosidase/sialyltransferase catalyzed synthesis of the sialyl-T core 1 structures **3a** and **3b**.

Transgalactosylation from lactose as a donor to Fmoc-Thr(GalNAcα1)-O*t*Bu (**1b**) catalyzed by the galactosidase resulted in the formation of the T antigen derivative Fmoc-Thr[Gal(β1-3)GalNAcα1]-O*t*Bu (**2b**). As the galactosidase also catalyzes the cleavage of the formed core 1 structure, the intermediate compound **2b** is capped in situ by subsequent sialylation yielding Fmoc-Thr[Neu5Ac(α2-3)Gal(β1-3)GalNAcα1]-O*t*Bu (Sialyl-T-core 1-Thr, **3b**). In contrast to **2b**, the conjugate **3b** is not affected by the galactosidase.

For economic reasons, first experiments towards an optimization of the one-pot reaction described above were carried out using benzyl-α-*N*-acetylgalactosamine (GalNAc[α1-OBn], **1a**) as a model compound. As a first step, pH dependencies of activity and stability of both enzyme preparations were determined. Whereas the sialyltransferase activity optimum at pH 6.5 differs considerably from the activity optimum of the galactosidase (pH 4.3), stabilities of both enzymes are highest close to neutral conditions. Sialyltransferase is far less stable than the galactosidase: half-lives of

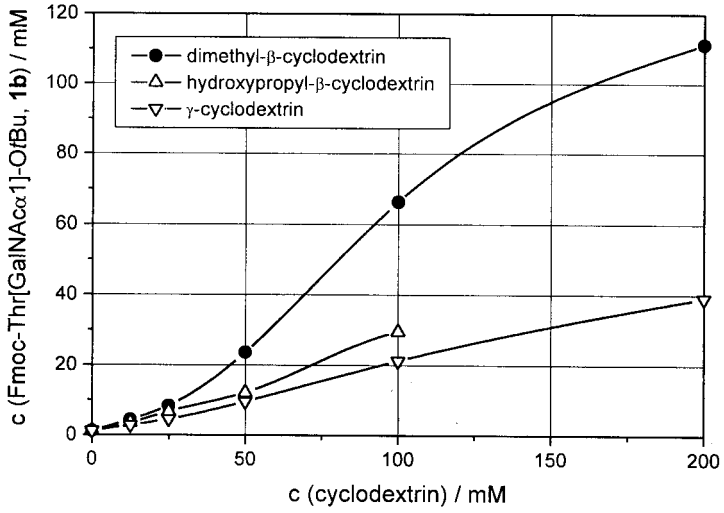

Figure 2. Enhancing the solubility of Fmoc-Thr(GalNAcα1)-O*t*Bu (**1b**) by the addition of cyclodextrins.

Table 1. Kinetic data of the transgalactosylation reaction employing lactose as a donor

Substrate	K_M (mM)	Relative v_{max} (–)
GalNAc(α1-Obn) (**1a**)	50±14	1
Fmoc-Thr(GalNAcα1)-O*t*Bu (**1b**)	17±5	0.25
Lactose	78±26	

enzyme activity were determined at 1.8 days (sialyltransferase in 50 mM MES, pH 6.5, 1 mg/mL BSA, 20°C) and 11.6 days, respectively (galactosidase in 100 mM MES, pH 6, 1 mg/mL BSA, 20°C). As stability of the transferase seems to be critical, all further reactions were carried out at pH 6.5 and 20°C. Alkaline phosphatase was added as a third enzyme to the one-pot reactions in order to prevent product inhibition of the sialyltransferase by CMP.[20]

On analytic scale, the one-pot reaction leading to Neu5Ac(α2-3)Gal(β1-3)GalNAc(α1-OBn) (**3a**) as a product was optimized to 90% yield. The intermediate Gal(β1-3)GalNAc(α1-OBn) (**2a**) could not be detected at any stage of reaction progress. For structural analysis, a preparative synthesis was carried out under the same conditions except the sialyltransferase preparation. Using the diafiltrate instead of the pure enzyme, the reaction still yielded 80% of the product **3a**. Its structure was confirmed by comparison to literature data.[21]

First attempts to adapt this procedure to the protected amino acid glycan **1b** suffered from its low solubility in water. Since the use of organic cosolvents rapidly deactivates the enzymes, cyclodextrins were added as complexing agents.[22–25]

β-Cyclodextrins are sufficiently large to enclose extended aromatic systems like the Fmoc-residue. Methylated cyclodextrins usually form more stable complexes than the unsubstituted cyclodextrins. Therefore 2,6-di-*O*-methyl-β-cyclodextrin was used.[26,27] In addition, hydroxypropyl-β-cyclodextrin and γ-cyclodextrin were investigated. Fig. 2 shows the enhanced solubility of substrate **1b** as a function of cyclodextrin concentrations. The best result was actually obtained with the dimethyl derivative. The cavity of the γ-cyclodextrin seems to be too large for efficient complexation, whereas hydroxypropyl-β-cyclodextrin shows poor inclusion properties and limited solubility.

Kinetic characterization of the galactosidase catalyzed transgalactosylation from lactose to Fmoc-Thr(GalNAcα1)-O*t*Bu (**1b**) showed that the cyclodextrin complex is accessible for the enzyme. Although the K_M-value of 17 mM for the acceptor **1b** is even lower than that for the model compound GalNAc(α11-OBn) (**1a**), the enzyme shows less activity towards **1b**. In Table 1, the kinetic parameters are presented.

In contrast to the syntheses with the model compound **1a**, another peak could be detected on HPLC during the course of reaction in this case. Most likely, it represents a regioisomer of the core 1 glycan **2b**, though unfortunately, we could not isolate and characterize it. This assumed lack in regioselectivity of the galactosidase does not affect the Fmoc-Thr[Neu5Ac(α2-3)Gal(β1-3)GalNAcα1]-OtBu (**2b**) synthesis, because a regioisomer cannot be converted further by the sialyltransferase. The core 1 intermediate itself, which was observed during conversions with the galactosidase as a single enzyme, could not be found at any stage of the reaction.

The optimized reaction yielded 67% of Sialyl-T-core 1-Thr (**3b**) on an analytical scale. In order to reduce enzyme consumption, preparative batches were carried out with lower enzyme activities yielding 50% of the trisaccharide **3b**. In all reactions with Fmoc-Thr(GalNAcα1)-O*t*Bu (**1b**) as a substrate, the purified sialyltransferase was used instead of the crude preparation.

The course of a representative preparative synthesis is shown in Fig. 3. Although 20 mM of **1b** were added, the starting concentration was lower due to precipitation of the acceptor substrate after dilution of the stock solution. After 16 h, the remaining substrate was dissolved completely.

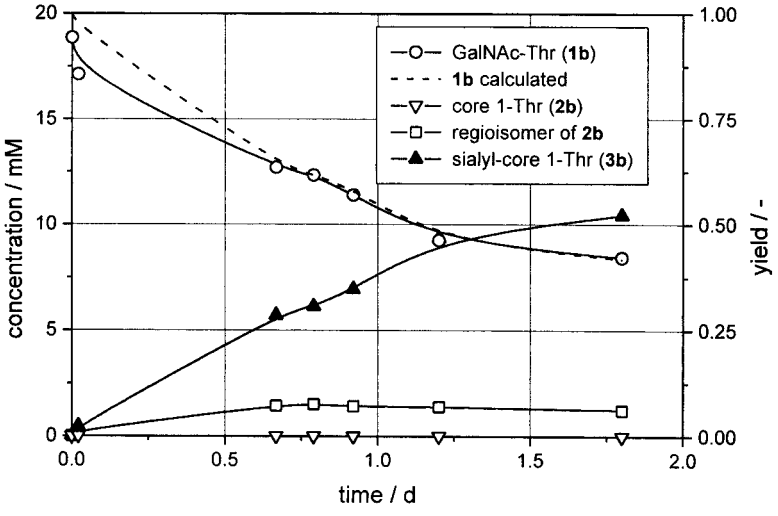

Figure 3. Course of reaction of the one-pot synthesis yielding sialyl-core 1-Thr **3b**.

The product **3b** was purified by preparative HPLC. Its structure was confirmed by NMR and mass spectroscopy. In total 100 mg of the trisaccharide **3b** were isolated.

Conclusion

In summary, we have enhanced the solubility of a protected amino acid glycan up to 100-fold by the use of cyclodextrins. This improvement enabled us to carry out an enzymatic glycosylation without the aid of organic cosolvents. The optimized one-pot reaction employing a β-galactosidase in combination with an α2,3-sialyltransferase opens up a way to readily synthesize sialylated core 1 amino acid conjugates that are potentially interesting as building blocks for the synthesis of complex glycopeptides.

Experimental

Materials

2,6-Di-*O*-methyl-β-cyclodextrin was kindly donated by Wacker AG, Germany. Alkaline phosphatase, bovine serum albumin, galactosidase, Gal(β1-3)GalNAc(α1-OBn) (**2a**) and GalNAc(α1-OBn) (**1a**) were obtained by Sigma, Germany. CMP-Neu5Ac was prepared at the Forschungszentrum Jülich according to previously published procedures.[28–30] YM ultrafiltration membranes were purchased from Amicon, Germany.

Standard enzyme assays

In order to determine galactosidase activity, hydrolysis of pnp-galactose was measured. (Assay conditions: 5 mM pnp-Gal in 0.1 mL citrate buffer, 100 mM, pH 4.3, 37°C.) The reaction is stopped by adding 0.9 mL glycine buffer (250 mM, pH 10). Sialyltransferase activity was determined with Gal(β1-3)GalNAc(α1-OBn) (**2a**) as an acceptor substrate. (Assay conditions: 1 mM **2a**, 1 mM CMP-Neu5Ac, 1 mg/mL BSA, 50 mM MES buffer, pH 6.5, 37°C.)

Ultrafiltration of sialyltransferase

First, baculo viruses were removed by passing 250 mL of cell culture supernatant through an ultrafiltration membrane (300 kDa cut off). After that, a tight ultrafiltration membrane with a 10 kDa cut off was used for diafiltration. The volume was exchanged ten times with MES buffer (100 mM, pH 6.5) and concentrated 30-fold. Finally, 3 U of sialyltransferase activity were obtained.

Synthesis of Neu5Ac(α2-3)Gal(β1-3)GalNAc(α1-OBn) (3a). Preparative synthesis was performed on a 2 mL scale with the following reactant concentrations: 0.5 M lactose (324 mg, 1 mmol), 20 mM GalNAc-(α1-OBn) (**1a**, 12.5 mg, 0.06 mmol), 30 mM CMP-Neu5Ac (38.2 mg, 0.06 mmol), 0.5 mg/mL bovine serum albumin, 0.5 U/mL galactosidase, 0.1 U/mL sialyltransferase, 10 U/mL calf intestine alkaline phosphatase, 50 mM MES buffer, pH 6.5, 20°C. Reaction yield was 80%. The product was purified by preparative HPLC (RP-18 column, CS-Chromatographieservice, Germany; water/acetonitrile 88/12-v/v; 0.1% trifluoro acetic acid) with a yield of 78%. 18 mg of **3a** were isolated. Analysis of reaction progress was performed by reversed phase HPLC (Lichrosorb RP-18, Merck, Germany; 25°C; same conditions as preparative HPLC).

Synthesis of Fmoc-Thr[Neu5Ac(α2-3)Gal(β1-3)GalNAcα1]-O*t*Bu (3b). A stock solution of 100 mM Fmoc-Thr(GalNAcα1)-O*t*Bu (**1b**, 300 mg, 0.5 mmol) and 200 mM 2,6-di-*O*-methyl-β-cyclodextrin (1330 mg, 1 mmol) in 50 mM MES buffer, pH 6.5, was prepared by mixing for 2 h. A representative synthesis on a 5 mL scale was carried out with the following reactant concentrations: 0.5 M lactose (810.7 mg, 2.5 mmol), 20 mM **1b** (60.1 mg, 0.1 mmol), 40 mM dimethyl-β-cyclodextrin (266.3 mg, 0.2 mmol), 25 mM CMP-Neu5Ac (95.5 mg, 0.15 mmol), 0.5 mg/mL bovine serum albumin, 1 U/mL galactosidase, 0.2 U/mL sialyltransferase, 10 U/mL calf intestine alkaline phosphatase, 50 mM MES buffer pH 6.5, 20°C. During the synthesis on analytical scale, additional sialyltransferase (50 mU/mL) was added after 2 days. Analysis of reaction progress was performed by reversed phase HPLC (Lichrosorb RP-18, Merck, Germany; 25°C; 40% MeCN; 0.1% trifluoro acetic acid; 0.5 mL/min). Syntheses were carried out on a 12 mL scale in total and yielded about 50% in all cases. The product was purified by preparative HPLC with a water/acetonitrile gradient (0 min: 10% MeCN; 120 min: 20% MeCN; 180 min: 20% MeCN; 195 min: 40% MeCN; 210 min: 40% MeCN; 225 min: 100% MeCN). 100 mg of **3b** were purified in total.

The structure of sialyl-T-core 1-Thr (**3b**) was analyzed by NMR and MALDI-TOF-MS:

400-MHz-NMR (^1H–^1H-COSY, CD_3OD), δ(ppm): 7.86–7.34 (m, $8H_{aromat.}$ Fmoc), 4.86 (d, 1H, H-1, $J_{H\text{-}1,H\text{-}2}$=3.8 Hz), 4.54 (d, 2H, CH_2 Fmoc, $J_{CH2\ Fmoc,H\text{-}9\ Fmoc}$=6.5 Hz), 4.51–4.37 (m, 2H, H-2, H-1′, $J_{H\text{-}1',H\text{-}2'}$=7.6 Hz), 4.32 (t, 1H, H-9 Fmoc, $J_{CH2\ Fmoc,H\text{-}9\ Fmoc}$=6.5 Hz), 4.20–4.17 (m, 2H, H-4, T^{α}), 4.04 (dd, 1H, H-3′, $J_{H\text{-}3',H\text{-}2'}$=9.4 Hz, $J_{H\text{-}3',H\text{-}4'}$= 2.6 Hz), 3.96–3.84 (m, 3H, H-3, H-4′, H-9a″), 3.81–3.68 (m, 10H, H-5, H-6a, H-6b, H-5′, H-6a′, H-6b′, H-4″, H-6″, H-7″, H-9b″), 3.66–3.60 (m, 1H, H-5″), 3.59–3.50, (m, 3H, H-2′, H-8″, T^{α}), 2.90 (d, 1H, $H\text{-}3_{eq}$″, $J_{H\text{-}3eq'',H\text{-}3ax''}$= 10.9 Hz), 2.05, 2.04 (2*s, 6H, CH_3CON), 1.76 (t, 1H, $H\text{-}3_{ax}$″, $J_{H\text{-}3ax'',H\text{-}3eq''}$=$J_{H\text{-}3ax'',H\text{-}4''}$=10.9 Hz), 1.48 (s, 9H, $C(CH_3)_3$), 1.40, 1.31 (d, 3H, T^{α}, $J_{T\gamma,T\beta}$=6.2 Hz).

100.6-MHz-NMR (^{13}C–^1H-COSY, CD_3OD), δ(ppm): 175.5, 174.8, 173.9, 171.1 (C═O), 159.3 (C═O urethane), 145.3, 142.7 ($C_{quart.}$ Fmoc), 128.9, 128.2, 126.1, 121.0 ($C_{tert.}$ Fmoc), 106.3 (C-1′), 100.9 (C-1), 83.5 ($C(CH_3)_3$), 78.9 (C-3), 77.7 (C-3′), 76.7 (T^{α}), 76.3 (C-8″), 74.9 (C-5″), 72.7 (C-4′), 71.4, 69.4 (H-6″, H-7″), 70.7 (C-2′), 70.1 (C-4), 69.1 (C-4″), 67.9 (CH_2 Fmoc), 66.7 (C-9″), 63.0, 62.9 (C-6, C-6′), 61.03 (T^{α}), 55.9, 54.1 (C-5, C-5′), 49.7-48.4 (C-2, C-9 Fmoc), 42.2 (C-3″), 28.4 ($C(CH_3)_3$), 23.6, 22.6 (CH_3CON), 19.8 (T^{γ}).

MALDI-TOF-MS (cca, positive). 1093.1 ($M+K^+$, calcd 1093.1), 1077.4 ($M+Na^+$, calcd: 1077.1), 1026.6 ($M-t\text{Bu}+Na^+ +Li^+$, calcd 1027.1), 1021.2 (M−*t*Bu+

$Na^+ + H^+$, calcd 1021.1) 784.7 (M−Neu5Ac+Na^+, calcd 784.8), 773.8 (M^+−Neu5Ac−$2H^+ + 2Li^+$, calcd 773.8), 735.8 (M−Neu5Ac−*t*Bu+$Na^+ + Li^+ + H^+$, calcd 735.8), 729.9 (M−Neu5Ac−*t*Bu+$Na^+ + 2H^+$, calcd 729.8).

References

1. Varki, A. *Glycobiology* **1993**, *3*, 97–130.
2. Fukuda, M. *Bioorg. Med. Chem.* **1995**, *3*, 207–215.
3. Kunz, H.; Schultz, M. *Glycopeptides and Related Compounds*; Large, D. G., Warren, C. D., Eds.; Marcel Dekker: New York, 1997, pp 23–78.
4. Brockhausen, I. In *Biosynthesis of O-Glycans of the N-Acetylgalactosamine-α-Ser/Thr linkage type*, Montreuil, J., Vliegenthart, J. F. G., Schachter, H., Eds.; Elsevier: Amsterdam, 1995; Vol. 29(a), pp 201–260.
5. Sears, P.; Wong, C. H. *Cell. Mol. Life Sci.* **1998**, *54*, 223–252.
6. Herrmann, G. F.; Ichikawa, Y.; Wandrey, C.; Gaeta, F. C. A.; Paulson, J. C.; Wong, C.-H. *Tetrahedron Lett.* **1993**, *34*, 3091–3094.
7. Kren, V.; Thiem, J. *Angew. Chem., Int. Ed. Engl.* **1995**, *34*, 893–895.
8. Dudziak, G.; Zeng, S.; Berger, E. G.; Gallego, R. G.; Kamerling, J. P.; Kragl, U.; Wandrey, C. *Bioorg. Med. Chem. Lett.* **1998**, *8*, 2595–2598.
9. Gambert, U.; Thiem *Eur. J. Org. Chem.* **1999**, 107–110.
10. Cantacuzene, D.; Attal, S. *Carbohydr. Res.* **1991**, *211*, 327–331.
11. Suzuki, K.; Fujimoto, H.; Ito, Y.; Sasaki, T.; Ajisaka, K. *Tetrahedron Lett.* **1997**, *38*, 1211–1214.
12. Mendicino, J.; Sivakami, S.; Davila, M.; Chandrasekaran, E. V. *J. Biol. Chem.* **1982**, *257*, 3987–3994.
13. Furukawa, K.; Roth, S. *Biochem. J.* **1985**, *227*, 573–582.
14. Brockhausen, I.; Moller, G.; Pollex-Krüger, A.; Rutz, V.; Paulsen, H.; Matta, K. L. *Biochem. Cell. Biol.* **1992**, *70*, 99–108.
15. Liebe, B.; Kunz, H. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 618–621.
16. Liebe, B.; Kunz, H. *Helv. Chim. Acta* **1997**, *80*, 1473–1482.
17. Sadler, J. E.; Rearick, J. I.; Paulson, J. C.; Hill, R. L. *J. Biol. Chem.* **1979**, *254*, 4434–4443.
18. Chang, M. L.; Eddy, R. L.; Shows, T. B.; Lau, J. T. Y. *Glycobiology* **1995**, *5*, 319–325.
19. Wandall, H. H.; Hassan, H.; Mirgorodskaya, E.; Kristensen, A. K.; Roepstorff, P.; Bennett, E. P.; Nielsen, P. A.; Hollingsworth, M. A.; Burchell, J.; Taylor-Papadimitriou, J.; Clausen, H. *J. Biol. Chem.* **1997**, *272*, 23503–23514.
20. Unverzagt, C.; Kunz, H.; Paulson, J. C. *J. Am. Chem. Soc.* **1990**, *112*, 9308–9309.
21. Pollex-Krüger, A.; Meyer, B.; Stuike-Prill, R.; Sinnwell, V.; Matta, K. L.; Brockhausen, I. *Glycoconjugate J.* **1993**, *10*, 365–380.
22. Matahira, Y.; Tashiro, A.; Sato, T.; Kawagishi, H.; Usui, T. *Glycoconjugate J.* **1995**, *12*, 664–671.
23. Murata, T.; Itoh, T.; Hayakawa, Y.; Usui, T. J. *Biochemistry* **1996**, *120*, 851–855.
24. Zelinski, T.; Kula, M.-R. *Biocat. Biotrans.* **1997**, *15*, 57–74.
25. Zelinski, T.; Liese, A.; Wandrey, C.; Kula, M.-R. *Tetrahedron: Asymmetry* **1999**, *10*, 1681–1687.
26. Wenz, G. *Angew. Chem., Int. Ed. Engl.* **1994**, *33*, 803–822.
27. Casu, B.; Reggiani, M.; Sanderson, G. R. *Carbohydr. Res.* **1979**, *76*, 59–66.
28. Simon, E. S.; Bednarski, M. D.; Whitesides, G. M. *J. Am. Chem. Soc.* **1988**, *110*, 7159–7163.
29. Kragl, U.; Klein, T.; Vasic-Racki, D.; Kittelmann, M.; Ghisalba, C.; Wandrey, C. *Ann. N.Y. Acad. Sci.* **1996**, *799*, 577–583.
30. Dudziak, G.; Fey, S.; Hasbach, L.; Kragl, U. *J. Carbohydr. Chem.* **1999**, *18*, 41–49.