Chemical synthesis using enzymatically generated building units for construction of the human milk pentasaccharides sialyllacto-N-tetraose and sialyllacto-N-neotetraose epimer

Dirk Schmidt and Joachim Thiem*
sialylated structures, enzymatic procedures are still considerably superior to classical chemical sialylations with respect to both stereochemical outcome and preparative input.

The use of both procedures in a synergistic mode should also be considered. One of the general approaches ideally suited in such cases is the block synthesis method. Moreover, in recent years a number of combined chemical and chemoenzymatic syntheses have been reported [1-4].

As a proof of principle, we were interested to employ some trisaccharide building units previously obtained by enzymatic routes in block syntheses en route to interesting structures. To this end, two human milk pentasaccharides of prominent importance, sialyllacto-N-tetraose (1) and an epimer of sialyllacto-N-neotetraose (2) (Figure 1) were selected as target molecules. Both these pentasaccharides, Neu5Acα2-3Galβ1-3GlcNAcβ1-3Galβ1-4Glc (1) and Neu5Acα2-6Galβ1-4GlcNAcβ1-4Galβ1-4Glc (2), are dominant constituents of complex human milk oligosaccharides (Figure 1). They are considered to play a major role in immuno defense against bacterial and viral infections in the gastrointestinal tract of infants [5]. It is thought that they effectively inhibit bacterial adhesion to epithelial surfaces and so block the first stages of infection processes. Thus, these human milk oligosaccharides are considered as soluble receptor analogues of epithelial cell surfaces [6].

Results and Discussion

Previously, we reported the chemoenzymatic synthesis of the 3-sialylated lactosamine derivative 3 obtained by the enzymatic β-galactosylation of the 2-azidothioglucoside with p-nitrophényl β-galactopyranoside and β-galactosidase (Bovin testes). The subsequent transsialylation was carried out with p-nitrophényl sialoside (pNp-αNeu5Ac) and either sialidase from Salmonella typhimurium or from Newcastle disease virus [7]. Recently, a more effective higher yielding transfer has been reported in which sialylation with recombinant transsialidase (Trypanosoma cruzi) gave the trisaccharide 3 in 32% yield [8]. Treatment of 3 with methanol and acidic ion exchange resin led to the methyl ester (for the method cf. lit. [9]) which was then peracetylated to give trisaccharide 4 as the donor building block.

For formation of the disaccharide acceptor 6, a straight-forward three-step standard reaction sequence was used [10]. Methyl β-lactoside was isopropyldenated at 3′,4′-position with dimethoxypropane and p-toluene sulfonic acid in DMF/acetone. Peracetylation (Ac₂O/Py) and subsequent cleavage of the isopropylidene group with 80% acetic acid at 80 °C gave the diol acceptor 6. Since it is known that in galactopyranosyl structures the nucleophilicity of 3-OH considerably exceeds that of the 4-OH-group, further protecting group manipulations were not required.

Glycosylation of 4 by 6 catalyzed by N-iodosuccinimide and trifluoromethane sulfonic acid (as introduced by van Boom et al. [11]) gave the β,1-3-linked pentasaccharide 7 in 61% yield. About 5% of the corresponding α,1-3-linked compound and ca. 7% of the bis (β,1-3- and β,1-4-) linked octasaccharide were observed as side products and separated by chromatography but these were not further characterized. Reduction of the 2′′′-azido...
Scheme 1: Preparation of pentasaccharide 8. 1) MeOH, acidic ion exchange resin; 2) Ac₂O, pyridine; 3) 80% HOAc, 90 °C; 4) NIS, CF₃SO₂H, 61%; 5) NiCl₂•6H₂O, H₃BO₃, EtOH, then NaBH₄, EtOH and acidic workup.

A similar approach was employed for the synthesis of the protected epimer of sialyllacto-N-neotetraose 14. β-Galactosylation of 2-azidothioglucoside with p-nitrophenyl β-galactopyranoside and β-galactosidase (Bacillus circulans) gave the β,1-3-linked isolactosamine derivative. Further sialylation at position 3′-OH with pNp-αNeu5Ac and either sialidase from Vibrio cholerae or Clostridium perfringens afforded the α,2-6-sialylated trisaccharide 9 exclusively [7]. Later studies showed that 9 could be obtained in an enhanced yield of 32% by transsialylation with recombinant transsialidase (Trypanosoma cruzi) [8]. Formation of the methyl ester and peracetylation led to the trisaccharide donor building block 10.

Synthesis of the disaccharide acceptor in this case started from methyl β-lactoside, which was transformed into its 4′,6′-benzylidene-protected derivative 11 in almost quantitative yield by transacetalization with benzaldehyde dimethyldiacetal in acetonitrile under p-toluenesulfonic acid catalysis. Subsequent peracetylation with acetic anhydride/pyridine, selective cleavage of the benzylidene group with 80% acetic acid at 90 °C and finally treatment with tert-butylidiphenylsilyl chloride and imidazole in DMF afforded the disaccharide 12 (cf. references [14,15]).
After activation of the trisaccharide donor 10 with N-iodosuccinimide and trifluoromethanesulfonic acid, the disaccharide acceptor unit 12 could be glycosylated to give the β,1-4-linked pentasaccharide derivative 13 in 53% yield. In addition, the corresponding α,1-4-linked pentasaccharide was obtained in 8% yield.

Finally, the azido group was reduced by the nickel boride method with sodium borohydride, nickel chloride and boric acid [12,13]. During this step partial cleavage of the tert-butyldiphenylsilyl groups was also observed. Complete removal was achieved with trifluoroacetic acid in dichloromethane. For characterization purposes, peracetylation was carried out to give the completely protected pentasaccharide 14 in 67% yield (Scheme 2). As evident from a comparison of the $^1$H NMR data of 14 with the precursor tri- and disaccharide units 10 and 12, the novel characteristic doublet for the anomeric H-1″ of the β-GlcNAc unit at δ 5.12 ($J_{1″2″} = 8.2$ Hz) as well as the downfield shift $\Delta$δ 0.15 of H-4′ to δ 4.14 compared to 12 were in accord with structure of the target pentasaccharide.

**Conclusion**

In this contribution chemoenzymatically generated sialyl α,2-3- and sialyl α,2-6-glycosylated thiophenol 2-azido-lactose derivatives were employed as precursors for sialylated lactosaminide donor substituents in triflic acid/N-iodosuccinimide glycosylations. With easily accessible selectively unprotected lactose acceptor glycosides the pentasaccharide structures sialyllacto-N-tetraose and the epimer of sialyllacto-N-neotetraose could be obtained in good yields, and subsequently transformed into their

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**Scheme 2:** Preparation of pentasaccharide 14. 1) MeOH, acidic ion exchange resin; 2) Ac$_2$O, pyridine; 3) 80% HOAc, 90 °C; 4) TBDPSCI, imidazole, DMF; 5) NIS, CF$_3$SO$_2$H, 53%; 6) NiCl$_2$•6H$_2$O, H$_2$BO$_2$, EtOH, then NaBH$_4$, EtOH, then acidic workup; 7) CF$_3$CO$_2$H, CH$_2$Cl$_2$. 
peracetylated derivatives for structure elucidation. Thus, a combination of enzymatic and purely chemical procedures was shown to be advantageous in the preparation of complex oligosaccharides.

**Experimental**

For general methods cf. reference [16]. The NMR data for the saccharide rings in the pentasaccharides 7, 8, 13 and 14 are denoted according to the Roman numerals I-V from the reducing end, as depicted for compounds 8 and 14 (Figure 2):

Methyl O-(methyl-5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2-3)-O-(2,4,6-tri-O-acetyl-β-D-galactopyranosyl)-(1-3)-O-(4,6-di-O-acetyl-2-azido-2-deoxy-β-D-glucopyranosyl)-(1-3)-O-(2,4,6-tri-O-acetyl-β-D-galactopyranosyl)-(1-4)-2,3,6-tri-O-acetyl-β-D-galactopyranoside (7): Glycosylation was carried out as described for the synthesis of compound 13 from compound 4 (95 mg, 83 μmol) as donor and compound 6 (50 mg, 86 μmol) as acceptor. The pentasaccharide derivative 7 was obtained as a colorless amorphous solid; 83 mg (61%); [α]_D^20 = −3.5 (c 0.1, CHCl₃); 1H NMR (500 MHz, CDC13) δ 5.75 (dt, 1 H, H-3IV), 5.72 (d, 1 H, NH), 5.39 (d, J, = 2.8 Hz, 1 H, H-4II), 5.36 (dd, J₂, III = 9.3 Hz, 1 H, H-7IV), 5.15 (dd, J₂, III = 8.0 Hz, 1 H, H-2II), 4.99–4.97 (m, 2 H, H-2IV, H-2III), 4.96 (dd, J₁, II = 7.8 Hz, J₂, II = 9.6 Hz, 1 H, H-3II), 4.91 (dd, J₁, II = 8.0 Hz, J₂, II = 10.2 Hz, 1 H, H-2IV), 4.87 (dd, J₁, II = 2.6 Hz, H-2IV), 4.83 (dt, J₃III = 4.4 Hz, J₃III = 12.1 Hz, 1 H, H-4IV), 4.75 (dd, J₅, VI = 10.1 Hz, J₆, VI = 2.2 Hz, H-6IV), 4.62 (dd, 1 H, H-3III), 4.57 (dd, J₂, III = 10.0 Hz, J₃, IV = 2.8 Hz, 1 H, H-3IV), 4.51 (d, J₁, II = 7.8 Hz, 1 H, H-1IV), 4.49 (dd, 1 H, H-6IV), 4.39 (dd, J₁, II = 8.0 Hz, 1 H, H-1III), 4.37 (dd, J₁, II = 8.1 Hz, 1 H, H-1III), 4.29 (dd, J₈, V = 12.4 Hz, J₈, V = 2.4 Hz, H-9V), 4.24 (dd, J₆, VI = 12.6 Hz, J₆, VI = 6.6 Hz, 1 H, H-6III), 4.17 (dd, J₅, VI = 10.2 Hz, H-5V), 4.13 (d, 1 H, NH), 3.75 (s, 3 H, COOCH3), 4.00 (dd, J₈, V = 5.8 Hz, 1 H, H-9V), 3.72 (bt, 1 H, H-5V), 3.59 (m, 1 H, H-5I), 3.47–3.44 (m, 2 H, H-3II, H-6IV), 3.39 (dd, J₆, VI = 11.0 Hz, J₆, VI = 7.8 Hz, H-6IV), 2.61 (dd, J₃III, III = 12.4 Hz, J₃III, III = 4.8 Hz, 1 H, H-3III), 2.17–1.33 (15s, 45 H, 14 OAc, 1 NAc), 1.91 (dd, J₃III, III = 11.8 Hz, 1 H, H-5IIIIV), C₆H₇O₅N₄O₃ (1641.45): Found C; 49.33%; H, 5.59; N, 3.62. Calculated C; 49.02%; H, 5.65; N, 3.41. MALDI-TOF: 1664.44 (M+Na)^+; 1680.59 (M+K)^+.

Methyl O-(methyl-5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosylonate)-(2-3)-O-(2,4,6-tri-O-acetyl-β-D-galactopyranosyl)-(1-3)-O-(4,6-di-O-acetyl-2-azido-2-deoxy-β-D-glucopyranosyl)-(1-3)-O-(2,4,6-tri-O-acetyl-β-D-galactopyranosyl)-(1-4)-2,3,6-tri-O-acetyl-β-D-galactopyranoside (8): Reduction and peracetylation of compound 7 was carried out as described for 14. Thus, from 80 mg (49 μmol) of 7, 65 mg (81%) of 8 was obtained as a colorless amorphous solid; [α]_D^20 = −12.7 (c 0.5, CHCl3); 1H NMR (500 MHz, CDC13) δ 5.74 (dt, 1 H, H-3IV), 5.72 (d, 1 H, NH), 5.38 (dd, J₃, IV = 2.8 Hz, 1 H, H-4IV), 5.33 (dd, J₃, IV = 9.3 Hz, 1 H, H-7IV), 5.22 (dd, J₂, III = 9.6 Hz, 1 H, H-3III), 5.05 (dd, J₁, II = 8.0 Hz, 1 H, H-2IV), 4.99 (dd, J₁, II = 10.2 Hz, H-2IV), 4.97 (m, 2 H, H-3II, H-6IV), 4.95 (dd, J₁, II = 8.0 Hz, J₂, II = 9.6 Hz, 1 H, H-3III), 4.91 (dd, J₁, II = 7.9 Hz, 1 H, H-2IV), 4.86 (dt, J₃III = 4.6 Hz, J₃III = 12.0 Hz, 1 H, H-1IV).

**Figure 2:** Roman numbering of saccharide units in all pentasaccharides for NMR assignment.
Methyl O-(methyl-5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-α-D-glycero-D-galacto-2-nonulopyranosyluronate)-(2-6)-O-(2,3,4-tri-O-acetyl-β-D-galactopyranosyl)-(4-4′)-(3,6 di-O-acetyl-2-azido-2-deoxy-β-D-glucopyranosyl)-(4-1′)-(3,3-di-O-acetyl-2-acylaminod-β-D-galactopyranosyl)-(1-4)-O-2,3,6-tri-O-acetyl-β-D-glucopyranoside (13): A solution of trisaccharide 10 (68 mg, 0.012 mmol) and disaccharide 12 (56 mg, 0.010 mmol) in anhydrous toluene (2 mL) was cooled to −40 °C. N-iodosuccinimide (20 mg, 0.094 mmol), molecular sieves (4 Å, 200 mg) were added, and after cooling a saturated solution of trifluoromethane sulfonic acid in CCl₄ (ca. 2 M, 50 μL) was added with vigorous stirring. The mixture was gradually warmed over 2.5 h to −10 °C. Ethyl acetate (20 mL) was added and the reaction quenched by addition of a saturated aqueous NaHCO₃ solution (10 mL). After filtration through Celite, the phases were separated. The organic phase was washed with aqueous Na₂O₃ solution (10 mL), dried over MgSO₄, evaporated and the residue purified by flash chromatography with petroleum ether/ethyl acetate 2:1. Compound 7 was obtained as a colorless amorphous solid; 58 mg (53%). [α]D²⁰ = −21.6 (c 0.3, CHCl₃); ¹H NMR (500 MHz, CDCl₃) δ 7.70–7.24 (m, 10, Ph), 5.74 (dd, 1 H, H-4-V), 5.70 (dd, J₁,₂ = 10.2 Hz, 1 H, H-2-V), 5.44 (dd, J₁,₂ = 9.9 Hz, 1 H, H-1-V), 5.61 (dd, J₂,₃ = 9.9 Hz, 1 H, H-1-V), 5.62 (dd, J₂,₃ = 9.9 Hz, 1 H, H-1-V), 5.22 (t, J₂,₃ = 9.9 Hz, 1 H, H-1-V), 5.10 (dd, J₁₂,₁ = 8.0 Hz, 2 H, H-1-V), 4.93 (t, J₁₂,₁ = 9.9 Hz, 1 H, H-2-V), 4.87 (2d, 2 H, H-1-V, H-3-V), 4.85 (dd, J₁₂,₁ = 10.2 Hz, 1 H, H-4-V), 4.65 (dd, 1 H, H-1-V), 4.61 (dd, J₁₂,₁ = 10.2 Hz, 1 H, H-4-V), 4.75 (ddd, J₁₂,₁ = 1.9 Hz, 1 H, H-4-V), 3.89 (ddd, 1 H, H-3-V), 3.83 (m, 2 H, H-5-V), 3.78 (s, 3 H, OCH₃), 3.77 (t, J₁₂,₁ = 9.9 Hz, 1 H, H-1-V), 3.62 (ddd, J₁₂,₁ = 10.0 Hz, J₂,₃ = 5.5 Hz, J₂,₃ = 2.0 Hz, 1 H, H-5-V), 3.61 (dd, 1 H, H-6-V), 3.59 (dd, 1 H, H-5-V), 3.56 (dd, J₂,₃ = 10.2 Hz, 1 H, H-2-V), 3.33 (s, 3 H, CH₃), 2.69 (dd, J₃₆₋₃₉ = 12.7 Hz, J₄₋₈ = 4.6 Hz, 1 H, H-3-V), 2.15–1.36 (14s, 42 H, 13 OAc, 1 NAc), 2.03 (dd, J₁₃₋₄ = 12.0 Hz, 1 H, H-3-V), 1.01 (s, 9 H, Si(CH₃)₃). C₈₁H₁₀₀O₄₅Si (1837.81): Found: C, 53.89; H, 6.34; N, 2.66. Calculated C, 52.94; H, 5.92; 3.04. MALDI-TOF: 1860.80 (M+Na)⁺; 1876.91 (M+K)⁺. The α₁,α'-anomer of 7 was obtained as colorless syrup (9 mg, 8%) and not further characterized.
1 H, H-6H), 3.82 (m, 2 H, H-5II, 3.77 (s, 3 H, OCH3), 3.75 (t, J = 10.1 Hz, 1 H, H-4I), 3.65 (ddd, Jab = 9.8 Hz, Jac = 5.6 Hz, Jac = 1.8 Hz, 1 H, H-5I), 3.59 (m, 2 H, H-6H), 3.57 (dd, Jab = 9.8 Hz, Jac = 9.8 Hz, Jca = 9.8 Hz, 1 H, H-5I), 3.54 (m, 2 H, H-6H), 3.31 (s, 3 H, CH3), 2.70 (dd, Jab = 12.5 Hz, Jac = 4.4 Hz, 1 H, H-3eq), 2.17–1.33 (16s, 48 H, 14 OAc, 2 NAc), 2.01 (dd, Jab = 12.0 Hz, 1 H, H-3ax), 1.00 (s, 9 H, SiCCH3). C69H96N2O44 (1657.49): Found C, 49.86; H, 5.77; N, 1.65. Calculated C, 50.00; H, 5.84; N, 1.69. MALDI-TOF: 1680.39 (M+Na)\(^+\); 1696.59(M+K)\(^+\).

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