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Reversible derivatization of sugars with carbobenzyloxy groups and use of the derivatives in solution-phase enzymatic oligosaccharide synthesis

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ABSTRACT

Simple protocols for attaching and detaching carbobenzyloxy (Cbz) groups at the reducing end of sugars was developed. Briefly, lactose was converted into its glycosylamine, which was then acylated with carbobenzyloxy chloride in high overall yield. The obtained lactose Cbz derivative was used in sequential glycosylations using glycosyltransferases and nucleotide sugars in aqueous buffers. Isolation of the reaction products after each step was by simple C-18 solid-phase extraction. The Cbz group was removed by catalytic hydrogenolysis or catalytic transfer hydrogenation followed by in situ glycosylamine hydrolysis. In this way, a trisaccharide (GlcNAc-lactose), a human milk tetrasaccharide (LNnT), and a human milk pentasaccharide (LNFPIII) were prepared in a simple and efficient way.

Introduction

Of the biopolymers, oligosaccharides and polysaccharides are the most difficult to prepare by chemical synthesis. Nucleic acids and peptides can be routinely synthesized by stepwise solid-phase chemical synthesis, but analogous chemical synthesis of oligo- and polysaccharides has not yet reached the same stage of maturation. The major reason for this is that both α and β stereoisomers can be formed in classical organic synthesis coupling reactions between monosaccharides resulting in complex product mixtures already after a few steps, and also the fact that a comparatively large effort is required to prepare all the different protected monosaccharides needed [1].

Nature uses strictly regio- and stereospecific enzymes to produce oligo- or polysaccharides [2] without the use of protecting groups, and since many enzymes are now available through genetic engineering, they have often been used in synthesis of oligosaccharides. For example [3], a sialyl lewis x tetrasaccharide derivative was synthesized starting from a solid-phase linked monosaccharide, using, sequentially, the appropriate activated monomers/enzymes. After cleavage from the solid phase, a tetrasaccharide was isolated in 57% overall yield. Thus, solid-phase enzymatic oligosaccharide synthesis is possible, and might be a viable alternative in some cases. However, large excesses of expensive reagents (enzymes and activated monosaccharides) are needed to drive the solid-phase coupling reactions to completion.

Solution phase synthesis, where much less reagent excesses are needed, is for shorter oligomers a more realistic alternative and has been used in many cases, even on a large scale. However, a simple and rapid protocol for isolation of the reaction product after each step is essential. Isolation of an oligosaccharide product from a typical enzymatic reaction mixture is not simple, unless the product bears a specific group (“tag”) that enables it to be easily isolated. Boons et al. recently [4] reported use of charged tags in combination with ion-exchange resin adsorption/desorption in stepwise enzymatic synthesis of neutral oligosaccharides. Hindsgaul et al. [5] used oligosaccharides with hydrophobic tags for fast isolation of the enzymatic reaction products by reversed phase silica solid-phase extraction. Gangliosides and other glycolipids were assembled [6] using a similar approach. The hydrophobic tag principle was also used by us [7,8] in examples of enzymatic syntheses of oligosaccharides. The hydrophobic group was then attached either by reductive amination at the sugar reducing end or by conversion into the glycosylamine, acylation with diethyl square, and reaction with an alkylamine. The reductive amination-mediated linkage was however not easily cleaved, which was a disadvantage in some cases. The squarate-mediated linkage, on the other hand, could be cleaved to regenerate the free reducing end and was flexible in the choice of lipid. However, the overall squarate-mediated
attachment-cleavage procedure was somewhat laborious.

In an effort to simplify, we now report attachment of hydrophobic carbobenzyloxy (Cbz) groups to sugar reducing ends and use of the Cbz derivatives in stepwise enzymatic synthesis of oligosaccharides in solution. Facile removal of the Cbz group/glycosylamine hydrolysis after finished synthesis produced oligosaccharides with a free reducing end. The advantage of synthesizing an oligosaccharide with a free reducing end is that it provides a choice of many subsequent modifications, such as reduction, reductive amination with different amines, direct conjugation with macromolecules, etc.

Results and discussion

The conversion of reducing sugars into glycosylamines is a common way to create an N-nucleophilic center for site-specific functionalization (e.g. acylation). Reaction of sugars with either methanolic ammonia [9], aqueous ammonia/ammonium hydrogen carbonate [10,11], ammonium carbamate/methanol [12], or saturated aqueous ammonium hydrogen carbonate [13] have been reported to give glycosylamines or the corresponding carbamates in good yields. We used a slight modification (24 h reaction of a reducing sugar with saturated aqueous ammonia bicarbonate at 40°C and pH 9) of the Likosherstev procedure [13] to obtain a crude glycosylamine preparation suitable for acylation. In previous work, acryloyl chloride [14], fluorenlymethoxy carbonyl chloride [11,15], acylthiones [9], or fatty acid chlorides [10] have been reacted with glycosylamines to obtain the corresponding glycosylamides. We sought a reasonably cheap acylating agent that could be used in excess and that introduced a hydrophobic group which could be removed later in a simple way. Carbobenzyloxy chloride fulfilled these criteria. Aciylation of crude lactose glycosylamine with an excess of carbobenzyloxy chloride in aq sodium carbonate/dioxane gave, after purification by solid-phase extraction with C-18 silica, the lactose-Cbz derivative (72% overall yield from lactose (Scheme 1). Cbz-glycosylamides have been described before [9,16]. The H NMR spectrum of 3 in D2O at 25°C showed similar broadening of the H-1 signal as was noted for analogous compounds [8]. However, raising the temperature to 80°C gave a sharp H-1 doublet with J = 9.2 Hz, indicative of the expected β configuration.

The lactose Cbz-derivative was converted back to lactose either by catalytic hydrogenolysis (H2/Pd/C) in slightly acidic aqueous media or [17] by catalytic transfer hydrogenolysis (ammonium formate/Pd/C), demonstrating the reversibility of the Cbz derivatization.

In analogy with earlier work [7,8] we subjected the lactose-Cbz derivative to consecutive enzymatic glycosylations, with isolation of the product after each step by reversed-phase C-18 solid-phase extraction. Products other than the desired products were seldom observed in the enzymatic reaction mixtures. Minor amounts of unreacted starting oligosaccharide or glycosyl products (with crude enzymatic skills). A future increased availability of nucleotide sugars and enzymes would make this type of enzymatic approach even more attractive.

Conclusion

Reversible derivatization of lactose with a Cbz group enabled efficient and simple stepwise synthesis of neutral reducing oligosaccharides, utilizing the appropriate glycosyltransferases and nucleotide sugars. Isolation of glycosylation products after each step was by simple C-18 solid-phase extraction, and the final products after removal of the Cbz group were isolated in good yields.

Synthesis and purification of the pentasaccharide (three coupling steps) starting from the lactose-Cbz derivative required 4 days, most of this time being overnight coupling time. Compared to “traditional” stepwise chemical synthesis this is much faster and requires less laboratory skills. A future increased availability of nucleotide sugars and enzymes would make this type of enzymatic approach even more attractive.

Experimental

Materials and general methods: Concentrations were performed at reduced pressure (bath temperature < 40°C). 1H NMR spectra were recorded at 298 K for filtered D2O solutions with Agilent 400 or 500 MR spectrometers unless otherwise stated. Chemical shifts are reported in parts per million (ppm) and referenced to internal acetone (δH = 2.225, δC = 30.0). LC/MS was performed on an Agilent 1100/Waters Micromass ZQ instrument using a 3.0 × 50 mm C18 column and gradient elution with 5:95 acetonitrile/water (with 0.1% formic acid) to 95:5 acetonitrile/water (with 0.1% formic acid). The mass detector was operated in alternating negative and positive ion modes. TLC was performed on Silica Gel 60 F254 (Merck, Darmstadt, Germany) plates. Analytes (1–5 mg/mL solutions) were applied to the plates with the aid of a glass capillary, and the plates were briefly evacuated (1 Torr) to evaporate the application solvent. Elution was with X:3:3:2 EtOAc-MeOH-AcOH-water mixtures, where X was varied between 6 and 20, depending on the polarity of the analytes. After elution, the spots were visualized by UV light and/or by dipping in either 5% aqueous sulfuric acid or 0.5% ninhydrin in butanol, followed by heating. Solid phase extraction was performed with C-18 reversed-phase silica columns (Waters Bondapak HC18, 37–55 micron, 125 Å). Gel filtrations were performed on Bio-Gel P2 columns (fine, 2 × 50 cm, packed and eluted with 95:5 water-1-butanol). Reagents and solvents of high commercial quality were used without further purification unless otherwise stated. Uridine 5'-
diphosphogalactose (UDP-Gal), uridine 5′-diphospho-N-acetylglucosamine (UDP-GlcNAc), and bovine serum albumin (BSA) were from Sigma-Aldrich Chemical Co., (St. Louis, MO, USA). Guanosine 5′-diphospho-l-fucose ammonium salt (GDP-Fuc) was prepared by chemical synthesis essentially as described \[20\]. Lacto-N-neotetraose and LNFPIII reference material was from IsoSep AB (Tullinge, Sweden). Sigma-Aldrich Chemical Co (G-5507 Gal-T, dry powder). One unit (U) of Bovine milk tyllactosamine per minute at 37

\[\text{Conversion of lactose-Cbz (6) into lactose (1), trisaccharide GlcNAc-lactose (5), tetrasaccharide LNnT (7) and pentasaccharide LNFPIII (9). All yields are calculated from compound 3.}\]

\[\text{Scheme 2.}\]

at r. t. while a solution of fresh carboxbenzoyloxy chloride (0.60 mL, 4.2 mmol) was added. Analysis by TLC showed that, after 3 h, all starting material had disappeared. The mixture was concentrated to about half the volume (to remove most of the dioxane), diluted with water (10 mL) then washed with diethyl ether (20 mL). The ethereal layer was washed with water (5 mL). The combined aq layers were concentrated to approx. 15 mL, then applied to a C-18 column (15 g, wetted with methanol, then equilibrated with water). The column was eluted first with water (30 mL), then with methanol-water mixtures (20 mL each, from 10 to 60% methanol, 10% increments). The fractions were checked by TLC, appropriate fractions were pooled, evaporated to a small volume, and lyophilized to give as a colorless solid (172 mg, 72%). Crystals were obtained from cold (+4°C) water, m. p. 245–248°C (d.), [\(\alpha\)]D 3 = +3 (c = 0.25, methanol, 25°C). The material showed a strong positive ion at \(m/z\) 476.1 (M + H)+, also positive ion peaks at \(m/z\) 493.1 (M + Na)+ and 314.0 (loss of Gal). HRMS: Calc. for C\(_{30}\)H\(_{52}\)NNaO\(_{12}\)\(^+\) 498.1582. Found: 498.1587. NMR data: 1H (25°C), \(\delta\) 3.420 (m, 1H), 3.547 (dd, 1H, J = 7.9, 9.9, 3.610–3.800 (m, 9H), 3.900–3.950 (M, 2H), 4.452 (d, 1H, J = 7.8), 4.820 (bd, 1H), 5.150–5.220 (m, 2H), 7.390–7.470 (m, 5H); 13C, \(\delta\) 158.0, 135.9, 128.7, 128.4, 127.7, 102.8, 81.6, 77.7, 76.1, 75.3, 75.0, 72.4, 71.4, 70.9, 68.5, 67.4, 61.0, 59.8.

Deprotection of 3 to give lactose (1), method 1 (with H\(_2\)/Pd/C): A solution of 3 (30 mg, 0.063 mmol) in water (3 mL) was mixed with a suspension of Pd/C (10%, 25 mg) in methanol (3 mL) containing acetic acid (50 microL) in a 100 mL round-bottom flask. The flask was equipped with a septum, magnetically stirred and flushed with nitrogen
from a balloon-syringe combination, then flushed with hydrogen and left stirring under hydrogen at rt for 2 h, after which TLC showed complete disappearance of starting material, a major lactose spot, and a weak slow-moving spot. After flushing the reaction vessel with nitrogen, the mixture was filtered (5 μm disposable filter), and the filter was washed with water and methanol. The filtrate was evaporated, coevaporated with 1 x water, then taken up in water. This solution, according to TLC, was almost pure lactose, however, some slow-moving impurities remained. The solution was evaporated and the residue (26 mg) was purified by gel filtration to give pure lactose (15 mg, 70%), identical to an authentic sample.

De-protection of 3 to give lactose (1), method 2 (with ammonium formate/Pd/C): A solution of 3 (30 mg, 0.063 mmol) in water (3 mL) containing conc. aqueous ammonia (110 μmol, 2.0 mmol) and formic acid (80 μmol, 2.0 mmol) was mixed with a suspension of Pd/C (10%, 15 mg) in methanol (2 mL) and then stirred at r. t. while being monitored by TLC. The starting material had disappeared in less than 10 min, and there was a major glycosylamine spot (slow) and a minor lactose spot (faster). Another portion of formic acid (40 μmol) was added, after which there was a gradual (over several hours) transformation of glycosylamine into lactose. The mixture was left overnight at r. t., after which there was a gradual (over several hours) transformation of starting material and appearance of a major, slower spot and a minor, even slower spot (presumably the intermediate glycosylamine). After flushing with nitrogen, the mixture was filtered (5 μm disposable filter), and the filter was washed with water and methanol. The filtrate was evaporated to dryness, coevaporated once with water and then lyophilized. The residue (33 mg) was purified by gel filtration, which gave pure lactose (21 mg, 95%), indistinguishable from an authentic sample.

**Compound 5 (GlcNac-lactose):** A solution of 3 (60 mg, 126 μmol), UDP-GlcNAc (disodium salt, Mw 651.3, 150 mg, 230 micromol), and BSA (50 mg) in sodium caducate buffer (0.25 M, with 0.015 M MnCl2, pH 7.3, 12 mL) was mixed with GlcNAc-T (1.0 U, 2.5 mL of a 0.4U/mL stock solution), the mixture was slowly stirred at 35 °C for 24 h. Analysis by TLC showed emergence of a slower, sulfonic acid and ninhydrin-positive spot. At this point, the reaction appeared to be 75–80% finished, with remaining starting material 3 present. More UDP-GlcNAc (50 mg) and GlcNAc-T (1.0 mL) was added, and the mixture was stirred for another 16 h at 35 °C, after which a TLC detected only traces of starting material 3. The mixture was diluted with water (5 mL) and then applied onto a C-18 column (3.0 g, wetted with methanol, then equilibrated with water). The column was eluted first with water (10 mL), then with methanol-water mixtures (10 mL each, from 10 to 60% methanol, 10% increments). The fractions were checked by TLC (ethyl acetate-methanol-acetic acid-water 12:3:3:2), fractions containing the major product 4 were pooled, evaporated to a small volume, and lyophilized to give a white solid (72 mg). Analysis by LC-MS showed a major peak with a strong positive ion at m/z 678.2 (M + H)⁺, and a strong negative ion at 677.1 (M − H)−. The H- and 13C NMR spectra showed the expected signals. A solution of this material (18 mg) in water (2.5 mL) was mixed with a suspension of Pd/C (10%, 15 mg) in methanol (2 mL) containing acetic acid (20 microl), all in a 50 mL round-bottom flask, which was then equipped with a stirrer magnet and a septum, and stirred while flushed with first nitrogen, then hydrogen. The mixture was left stirring under a slight hydrogen overpressure at rt for 2 h, after which TLC showed complete disappearance of starting material and appearance of a major, slower spot and a minor, even slower spot (presumably the intermediate glycosylamine). After flushing with nitrogen, the mixture was filtered (5 μm disposable filter), and the filter was washed with water and methanol. The filtrate was mixed with another portion of acetic acid (20 microl), and was left at rt overnight in a glass vessel after which TLC indicated disappearance of the presumed glycosylamine spot. The solution was partially evaporated and applied onto a gel filtration column. Appropriate fractions were pooled, partially evaporated and then lyophilized to give pure (by TLC and NMR) 7 as a colorless powder (10 mg, 50%, calculated from 3) identical to an authentic sample of LNFPIII. Physical constants (NMR and MS) were as reported [18].

**Compound 9 (LNFPIII):** Compound 6 from the prep above (12 mg, 14.3 micromol) and GDP-fucosidium salt (13 mg, approx. 21 micromol) in sodium caducate buffer (0.10 M, with 0.015 M MnCl2, pH 7.5, 3.0 mL) was mixed with Fuc-T (100 μU, 0.10 mL of a 1 U/mL stock solution) and the mixture was stirred gently overnight at 35 °C, after which TLC indicated a complete reaction. The mixture was diluted with water (3 mL) and applied to a C-18 column (0.3 g, wetted with methanol, then equilibrated with water). The column was eluted first with water (4 mL), then with methanol-water mixtures (4 mL each, from 5 to 50% methanol, 5% increments). The fractions were checked by TLC. Appropriate fractions were pooled, partially evaporated, and lyophilized to give a white solid (18 mg) containing compound 7 and minor impurities. Analysis by LC-MS showed a peak with a strong positive ion at m/z 987.3 (M + H)⁺, and the H- and 13C NMR spectra showed the expected signals. This material (18 mg) in water (2.5 mL) was mixed with a suspension of Pd/C (10%, 20 mg) in methanol (2 mL) containing acetic acid (20 microl). After stirring under hydrogen at room temperature and atmospheric pressure for 1 h, analysis by TLC (ethyl acetate-methanol-acetic acid-water, 6:3:3:2) showed complete disappearance of starting material. The mixture was filtered with a 5 μm disposable filter, and the filter was washed with water and methanol. Since TLC detected traces of a glycosylamine-like spot, the filtrate was mixed with another portion of acetic acid (20 microL), and was left at rt overnight after which TLC indicated disappearance of this spot. The mixture was applied onto a gel filtration column. Appropriate fractions were pooled, partially evaporated and then lyophilized to give a residue (11 mg), which, according to TLC (ethyl acetate-methanol-acetic acid-water, 6:3:3:2) and H NMR in D₂O, contained LNFPIII [18] and traces of unreacted LNFPII (<10%). The estimated yield of pure LNFPIII was 41% calculated from compound 3, and 81% calculated from compound 6.

**Declaration of competing interest**

The authors declare that they have no known competing financial

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interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.carrres.2021.108272.

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