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Expedient syntheses of neoglycoproteins using phase transfer catalysis and reductive amination as key reactions‡

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Starting from peracetylated chloro- or bromo-glycosyl donors of *N*-acetylneuraminic acid, *N*-acetylglucosamine, glucose and lactose, the corresponding *p*-formylphenyl glycosides were synthesized stereospecifically under phase transfer catalysed conditions at room temperature in yields of 38–67%. After Zemplén de-*O*-acetylation, the formyl groups were directly and chemoselectively coupled to the lysine residues of bovine serum albumin by reductive amination using sodium cyanoborohydride. The conjugation reactions were followed as a function of time and under a series of different molar ratios of the reactants to provide glycoconjugates of varying degree of antigenicities. Thus, carbohydrate protein conjugates were made readily available using essentially two key reactions.

Keywords: sialic acid, glycoconjugate, phase transfer catalysis, reductive amination, aryl glycosides

Artificial carbohydrate protein conjugates, so-called neoglycoproteins, continue to attract the attention of biochemists and immunochemists because of the large number of events in which they are implicated. Among these, our group is particularly interested in some fundamental aspects of binding such as the evaluation of threshold sugar densities on glycoproteins for the expression of antigenicities. In order to evaluate the implications of the carbohydrate contents on the antigenicities of model neoglycoproteins, we prepared a number of simple glycoconjugates including sialic acid, *N*-acetylglucosamine, D-glucose, and lactose useful in serological studies.

The preparation of neoglycoproteins has been previously reviewed [1, 2]. Among the various methodologies, *p*-aminophenyl glycosides [3] and alditol derivatives [4, 5] still find widespread applications in spite of evident drawbacks. For instance, the diazotization reaction requires noxious reagents and is not chemoselective, while the isothiocyanate method requires labile thiophosgene reagent. Our experience in reductive amination [6] with both reducing sugars [7] and aglycons containing terminal aldehyde functionalities [8, 9] prompted us to utilize this methodology. It was also realized that efficient phase transfer catalysed (PTC) glycosylations [10–12] of *p*-hydroxybenzaldehyde followed by direct reductive amination of the *p*-formylphenyl glycosides should ensure expeditious approaches to neoglycoproteins (Fig. 1).

Results and discussion

Synthesis of p-formylphenyl glycosides by PTC

Although there has been a fair number of utilizations of PTC for the syntheses of aryl glycosides [10–14] including thioglycosides [12] and sialic acid α-glycosides [14], none of the previous examples reported the direct incorporation of an aldehyde functionality through glycosylation of *p*-hydroxybenzaldehyde. Moreover, except for the synthesis of a glycosyl azide [15], there was no report on the use of PTC conditions on a 2-acetamido-2-deoxy-glycosyl chloride such as **2**. Recent results [14] on the application of PTC to the synthesis of neuramidase substrates prompted us to describe our own results on the preparation of sialylated neoglycoproteins of biological interests [8, 9].

Thus, treatment of acetochloroneuraminic acid **1** with *p*-hydroxybenzaldehyde and tetrabutylammonium hydrogen sulfate (TBAHS) as phase transfer catalyst at room temperature afforded a 65% yield of the α-ketoside **5** as the sole

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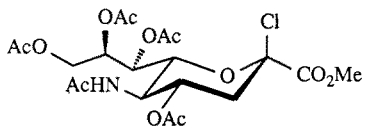

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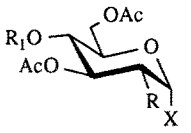
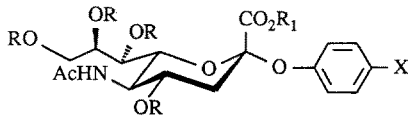
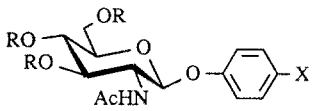


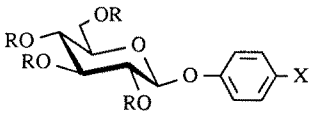
2 X = Cl, R = NHAc, R_1 = Ac
3 X = Br, R = OAc, R_1 = Ac
4 X = Br, R = OAc, R_1 = Gal(OAc)$_4$



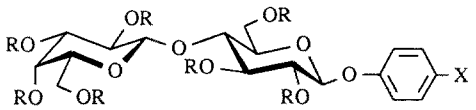
5 R = Ac, R_1 = Me, X = CHO
6 R = H, R_1 = Me, X = CHO
7 R = R_1 = H, X = CHO
8 R = R_1 = H, X = CH_2-NH-BSA



9 R = Ac, X = CHO
10 R = H, X = CHO
11 R = H, X = CH_2-NH-BSA



12 R = Ac, X = CHO
13 R = H, X = CHO
14 R = H, X = CH_2-NH-BSA



15 R = Ac, X = CHO
16 R = H, X = CHO
17 R = H, X = CH_2-NH-BSA

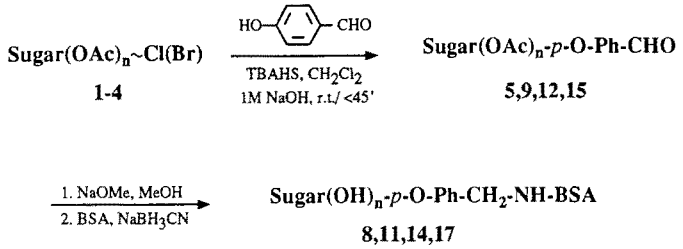


Figure 1. PTC syntheses of *p*-formylphenyl glycosides and reductive amination to BSA.

glycosidic product obtained. The method is therefore stereospecific. The major by-product was the known 2,3-dehydro derivative formed by elimination of hydrogen chloride from the glycosyl donor **1**. This method was preferred over the previous ones [11–14] using benzyltriethylammonium chloride as catalyst because the reaction with TBAHS was slightly faster and did not require refluxing conditions. The anomeric configuration of **5** was established using the well accepted empirical rules [16, 17] for which the H-4 and H-7 proton chemical shifts were observed at 4.96 and 5.35 ppm respectively. Zemplén de-*O*-acetylation followed by saponification of the methyl ester **6** (0.1 M NaOH) afforded the free aldehyde **7** in 84% overall yield.

When the above glycosylation procedure was applied to 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride **2**, the β-*N*-acetyl-D-glucopyranoside **9** was obtained in 67% yield. Only minor quantities of the 2-methyl-(3,4,6-tri-*O*-acetyl-1,2-dideoxy-α-D-glucopyrano)-[2,1,d]-oxazoline and hydrolysis by-products were observed (identified by $[\alpha]_D$ and ^{1}H-NMR). The oxazoline by-product was obviously formed by a double displacement reaction from chloride ions. The anomeric proton was shifted to 5.42 ppm ($J_{1,2}$ 8.1 Hz) and the anomeric carbon appeared at 97.7 ppm with a $J_{^{13}C\text{-}1,H\text{-}1}$ of 160.3 Hz consistent with a β-linkage. Treatment of **9** under the usual conditions with methanolic sodium methoxide afforded the crystalline aldehyde **10** in 98% isolated yield.

Attempts to synthesize the corresponding β-D-glucopyranoside **12** and β-D-lactoside **15** under a similar set of conditions were only met with limited success. Thus, starting from the homologous donors **3** and **4** using TBAHS and *p*-hydroxybenzaldehyde at room temperature, the peracetylated β-D-glycosides **12** and **15** were obtained in 38 and 45% yield respectively. The reaction was also stereospecific since only β-D-glycosides were formed. However, the reactions also furnished the respective peracetylated 2-acetoxy-D-glycals in 45 and 49% yield respectively (identified by m.p.,

$[\alpha]_D$, and ^{1}H-NMR). These side products resulted from dehydrohalogenation reactions by abstraction of the H-2 protons. These results are rather surprising because similar PTC reactions with other phenoxides gave much better yields (Roy, Tropper; unpublished results). Efforts to improve the yields using the analogous glycosyl chlorides failed; only very slow reactions (>2 days) were observed at room temperature and almost exclusive formations of the 2-acetoxy-D-glycals were obtained under refluxing conditions. Finally, Zemplén de-*O*-acetylation of both **12** and **15** afforded the free aldehydes **13** and **16** in essentially quantitative yields. The anomeric proton of **13** appeared as a doublet ($J_{1,2}$ 7.5 Hz) at 5.05 ppm and its anomeric carbon appeared at 99.6 ppm while the corresponding signals in **16** were observed at 5.32 ppm ($J_{1,2}$ 7.7 Hz) and at 99.1 ppm respectively.

Direct conjugation of the aldehydes **7**, **10**, **13** *and* **16** *onto BSA by reductive amination*

The direct reductive amination of the *p*-formylphenyl glycosides onto bovine serum albumin (BSA) using sodium cyanoborohydride was accomplished in 0.2 M phosphate buffer saline pH 7.0 at 37°C as described previously [8]. Different reactant molar ratios were used. Moreover, the reactions were followed as a function of time and aliquots were withdrawn at time intervals (Figs 2, 3) in order to obtain a series of neoglycoproteins having a wide distribution of sugar contents with different levels of antigenic expression. The sialic acid contents of the conjugates **8** were

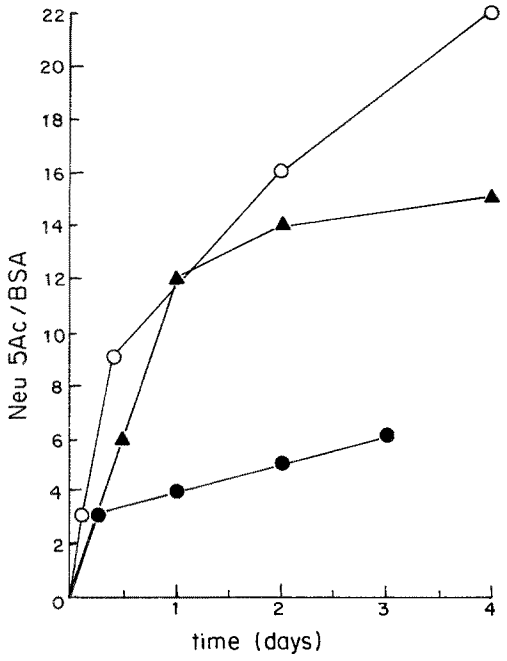


Figure 2. Time course of reductive amination of **7** with BSA in 0.2 M phosphate buffer pH 7.0, 37°C. **7**:NH_2:$NaBH_3CN$ at 4:1:9 (○), 3:1:7 (▲) and at 2:1:2 (●).

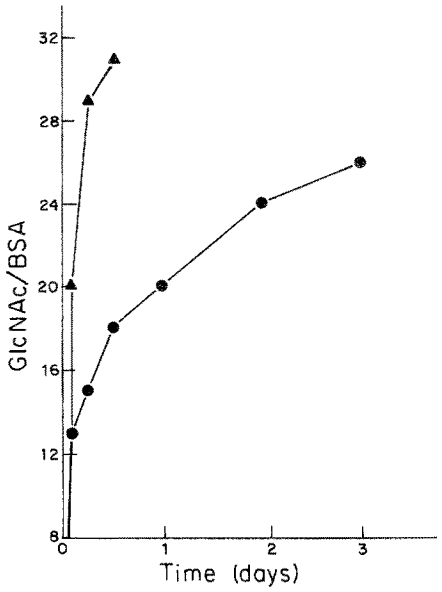


Figure 3. Time course of reductive amination of **10** with BSA in 0.2 M PBS pH 7.0, 37°C. Ratio of **10**:NH_2:$NaBH_3CN$ at 5:1:6 (▲) and at 2:1:2 (●).

measured by the resorcinol method [18] while for the conjugates **14** and **17** the phenol/sulfuric acid method of Dubois *et al.* [19] was used. In the case of the conjugate **11**, difficulties were encountered in the precise determination of the glucosaminide content because the protein was causing interferences in the colorimetric method [20]. Therefore, as preliminary results, we have partially characterized one of the conjugates **11** (5:1:6, see the Experimental section) by measuring the number of modified L-lysine residues by amino acid analysis (Fig. 3). It should be kept in mind however, that the number of glucosaminides on the protein might be higher than the modified lysine groups since two aldehyde residues can react on each primary amine of the lysines. No other amino acids were modified. It is interesting to mention that the rate and level of incorporation of the sialic acid glycoside **7** was lower than the neutral glycosides **10**, **11** and **16**. This was accounted for by the repulsive build up of negative charges on the acidic BSA protein as previously observed [8].

Because of the interference of the colorimetric assay with the *N*-acetylglucosaminides **10** mentioned above, accurate measurements of **10** on the BSA conjugate was also performed by amino acid analyses. For instance, the conjugate **11** (▲, Fig. 3) had 35, 43 and 50 lysines modified after 1, 2 and 4 days respectively (not indicated on the curve). Since all these conjugates were very antigenic with wheat germ agglutinin, they were not compared further.

Initial observations on the conjugate **11** clearly illustrate the effect of the sugar contents on the antigenicities. This is shown by the graded intensities of the precipitin bands obtained with **11** and wheat germ agglutinin (WGA) by

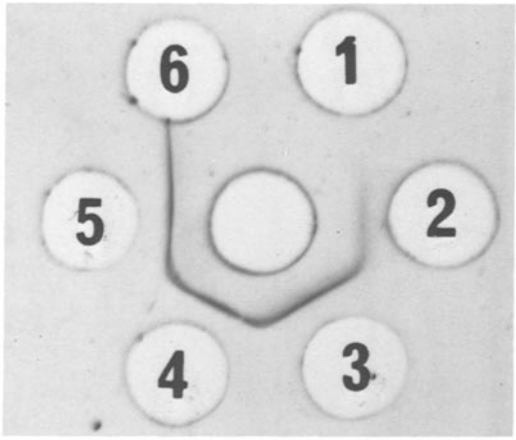


Figure 4. Agar double immunodiffusion of **11**. Middle well: WGA; well 1 to 5: conjugates (GlcNAc/BSA) obtained after 6 h (15 GlcNAc), 12 h (18 GlcNAc), 1 day (20 GlcNAc), 2 days (24 GlcNAc), and 3 days (26 GlcNAc) respectively; well 6: BSA control.

double immunodiffusion (Fig. 4). Conjugates obtained after 20 min, 1, 2, 4 and 6 h, although known to contain some of the derivative **10**, were not antigenic in double immunodiffusion tests. At a concentration of 1 mg ml^{-1}, the conjugate **11** obtained after 12 h (**10**:NH_2:$NaBH_3CN$, 2:1:2; 18 GlcNAc/BSA) showed a weak precipitin band. The bands were however very distinct at a GlcNAc/BSA ratio of $\geqslant 20$ ($\geqslant 1$ day). By contrast, the conjugates **8** having less than ≈ 15 sialic acid residues did not show any precipitin band with WGA. The β-D-lactoside conjugates **17** also showed precipitin bands of graded intensities in double immunodiffusion against *Ricinus communis* lectin. These phenomena have been substantiated by quantitative precipitation and ELISA experiments. Interestingly, ELISA (not shown) appeared to be more sensitive in these assays since conjugates having slightly lower GlcNAc/BSA ratios still showed binding capacities. Further studies are in progress to evaluate the serological properties and extent of antigenicities of these conjugates.

In conclusion, phase-transfer catalysis represents an expeditious route toward the synthesis of glycosides having reactive aldehyde functionalities which could be directly coupled to proteins by reductive amination. Protected forms of the aldehyde groups could give access to glycosides useful in block oligosaccharide syntheses.

Materials and methods

GENERAL METHODS

Melting points were determined on a Gallenkamp apparatus and are uncorrected. The ^{1}H- and ^{13}C-NMR spectra were recorded on a Varian XL-300 or a Varian Gemini 200 at 300 and 200 MHz respectively for protons and at 75.4 and 50.3 MHz respectively for carbons. The proton chemical shifts (δ) are given relative to internal chloroform (7.24 ppm) for $CDCl_3$ solutions, to internal acetone (2.216 ppm) for ^2H_2O solutions and to DMSO (2.50 ppm) for DMSO-d_6 solutions. The carbon chemical shifts are given relative to deuterochloroform at 77.0 ppm, to internal acetone (31.1 ppm) for ^2H_2O solutions and to DMSO-d_6 (39.5 ppm). The analyses were done as a first order approximation. Optical rotations were measured on a Perkin-Elmer 241 polarimeter and were run at 23–25°C for 0.5–1.0% solutions in chloroform unless otherwise stated. Mass spectra were recorded on a VG 7070-E spectrometer. Elemental analyses were performed by Guelph Chemical Laboratories Ltd (Ontario, Canada) or M-H-W Laboratories (Phoenix, AZ, USA). Thin-layer chromatography (TLC) was performed using Silica Gel 60-F254 plates and column chromatography on Silica Gel 60 (230–400 mesh, E. Merck No. 9385). The developed plates were sprayed or dipped with a solution of ceric sulfate (1%) and ammonium molybdate (2.5%) in 10% aqueous sulfuric acid and heated at ≈ 150°C. Purifications by Chromatotron were performed on a Harrison Research Chromatotron model 7924 using Silica Gel 60-F254 rotors (1, 2 or 4 mm thickness). All solvents and reagents used were reagent grade and, when required, further purifications were accomplished following published procedures [21]. Double immunodiffusion experiments were run in 1% agar (PBS) containing 2% PEG 8000 (Sigma Chemical Co., St Louis, MO, USA). The conjugates and WGA (Sigma) were at a concentration of 1 mg ml^{-1} PBS.

PTC SYNTHESIS OF *p*-FORMYLPHENYL GLYCOSIDES

Methyl (p-formylphenyl 5-acetamido-4,7,8,9-tetra-O-acetyl-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosid)onate (**5**)

A solution of *p*-hydroxybenzaldehyde (49 mg, 1.3 eq.) and tetrabutylammonium hydrogen sulfate (128 mg, 1.2 eq.) in 1 M NaOH (2 ml) was stirred at room temperature with a freshly prepared solution of **1** (158, 0.31 mmol) [8] in methylene chloride (2 ml) for ≈ 10 min. When TLC indicated complete transformation of **1**, the reaction mixture was diluted with 10 ml each of ethyl acetate and saturated aqueous sodium hydrogen carbonate. The organic layer was separated and washed with saturated sodium hydrogen carbonate (2 × 10 ml) followed by saturated sodium chloride (10 ml). It was dried (Na_2SO_4) and then concentrated to dryness at room temperature under vacuum. The oily residue was purified on a silica gel disk (Chromatotron) using ethyl acetate as eluent. The pure product **5** crystallized from benzene/hexane (120 mg, 65%) had m.p. 90.4–91.6°C, $[\alpha]_D$ +32.4°.

Analytical data calculated for $C_{27}H_{33}NO_{14}$: C, 54.45; H, 5.59; N, 2.35. Found: C, 54.36; H, 5.73; N, 2.31.

NMR data: ^{1}H, δ 9.90 (s, CHO), 7.81 (d, $J_{m,o}$ 8.7 Hz, H-meta), 7.15 (d, H-ortho), 5.36 (m, J_{8-9} 2.2, J_{8-9} 4.4 Hz, H-8), 5.35 (m, $J_{6,7}$ 1.4 Hz, H-7), 5.20 (d, $J_{5,NH}$ 10.0 Hz, NH),

4.96 (ddd, $J_{3a,4}$ 12.2 Hz, $J_{3e,4}$ 4.7 Hz, $J_{4,5}$ 10.3 Hz, H-4), 4.58 (dd, $J_{5,6}$ 10.8 Hz, H-6), 4.23 (dd, $J_{9,9}$ 10.3 Hz, H-9), 4.11 (ddd, H-5), 4.10 (dd, H-9′), 3.62 (s, CO_2Me), 2.71 (dd, J_{3a-3} 13.1 Hz, H-3e), 2.28 (dd, H-3a), 2.17, 2.10, 2.04, 2.03, 1.91 (5 s, OAc); ^{13}C, δ 191.2 (CHO), 171.0, 170.7, 170.5, 170.3, 170.1, 168.4 (C=O), 159.1 (C-ipso), 131.9 (C-para), 131.7 (C-meta), 118.8 (C-ortho), 99.4 (C-2), 73.5 (C-6), 68.6; 68.2, 67.0 (C-4,7,8), 61.9 (C-9), 53.1 (CO_2Me), 49.1 (C-5), 38.5 (C-3), 21.0, 20.8, 20.6, 2 × 20.5 (HN-, O-Ac).

Methyl (p-formylphenyl 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosid)onate (**6**)

Aldehyde **5** (119.3 mg, 0.200 mmol) was dissolved in methanol (10 ml) and the solution was cooled to 0°C. The pH of the solution was adjusted to 9 using 1 M NaOMe in methanol and the solution was stirred for 5 h at room temperature. The reaction was neutralized using H^+ resin and after filtration and evaporation a clear oil was obtained. The oil was dissolved in water and the solution was lyophilized. An amorphous white powder was obtained (80.0 mg, 93% yield). Compound **6** had m.p. 109.2–110.1°C, $[\alpha]_D$ +56.3° (H_2O).

NMR data ^1H (^2H_2O), δ 9.84 (s, CHO), 7.94 (d, $J_{m,o}$ 8.9 Hz, H-meta), 7.31 (d, H-ortho), 4.28 (dd, $J_{5,6}$ 10.5, $J_{6,7}$ 1.3 Hz, H-6, tentative), 4.00 (dd, $J_{4,5}$ 10.1 Hz, H-5, tent.), 3.58–3.87 (m, H-4, H-8, H-9,9′), 3.76 (s, CO_2Me), 3.59 (dd, $J_{7,8}$ 9.0 Hz, H-7, tent.), 2.85 (dd, $J_{3a,3e}$ 12.9, $J_{3e,4}$ 4.5 Hz, H-3e), 2.13 (dd, $J_{3a,4}$ 11.9 Hz, H-3a), 2.06 (NHAc); ^{13}C (^2H_2O), δ 194.6 (CHO), 174.6 (NAc), 169.3 (C=O), 158.6 (C-ipso), 131.8 (C-para, C-meta), 119.6 (C-ortho), 100.1 (C-2), 73.7 (C-6), 70.1 (C-8), 67.6 (C-4), 66.3 (C-7), 62.7 (C-9), 53.1 (CO_2Me), 51.1 (C-5), 39.6 (C-3), 21.7 (NAc).

p-Formylphenyl 5-acetamido-3,5-dideoxy-D-glycero-α-D-galacto-2-nonulopyranosidonic acid (**7**)

A solution of **3** (144.3 mg, 0.338 mmol) in 0.1 M NaOH was stirred at 0°C for 6 h. The reaction was quenched using H^+ resin and after filtration the solution was neutralized using 0.1 M NaOH. The solution was lyophilized to afford an amorphous powder (32.1 mg, 0.303 mmol, 90% yield). Compound **7** had m.p. 180–184°C (dec.), $[\alpha]_D$ +72.4° (H_2O). FAB-MS (neg) for $C_{18}H_{22}NO_{10}$: M^- (412, 8.4%), M^--OPhCHO (311, 7%).

NMR data ^1H (^2H_2O), δ 9.83 (s, CHO), 7.92 (d, $J_{m,o}$ 8.8 Hz, H-meta), 7.32 (d, H-ortho), 4.16 (dd, $J_{5,6}$ 10.4, $J_{6,7}$ 1.5 Hz, H-6, tent.), 3.97 (dd, $J_{4,5}$ 9.8 Hz, H-5, tent.), 3.59–3.88 (m, 5-H, H-4, H-7, H-8, H-9,9′), 2.87 (dd, $J_{3a,3e}$ 12.7, $J_{3e,4}$ 4.6 Hz, H_{3e}), 2.06 (s, NHAc), 2.03 (dd, $J_{3a,4}$ 11.9 Hz, H-3a); ^{13}C (^2H_2O), δ 194.7 (CHO), 174.6 (NAc), 172.6 (CO_2^-), 159.7 (C-ipso), 131.6 (C-para, C-meta), 119.8 (C-ortho), 101.9 (C-2), 73.5 (C-6), 71.1 (C-8), 67.8 (C-4), 67.3 (C-7), 62.3 (C-9), 51.3 (C-5), 40.5 (C-3), 21.6 (NAc).

p-Formylphenyl 2-acetamido-3,4,6-tri-O-acetyl-2-deoxy-β-D-glucopyranoside (**9**)

The chloride **2** (102 mg, 0.28 mmol), *p*-hydroxybenzaldehyde (68 mg, 0.56 mmol, 2 eq.) and tetrabutylammonium hydrogen sulfate (93 mg, 0.28 mmol, 1 eq.) were dissolved in 1 M NaOH (1 ml) and methylene chloride (1 ml). The mixture was vigorously stirred at room temperature until TLC ($CHCl_3$/ETOAc/*n*-PrOH, 25/5/3 by vol) indicated complete transformation of the chloride **2** (≈40 min). Ethyl acetate (15 ml) was added to the reaction mixture and the recuperated organic phase was washed with 1 M NaOH (3 × 15 ml), water (2 × 15 ml) and once with saturated NaCl. The dried organic phase (Na_2SO_4) was evaporated under vacuum and the residue purified by preparative TLC using the above solvent as eluent. Compound **9** was obtained from the silica gel by extraction with ethyl acetate to give 84 mg, 67% yield. Crystallization from ethanol gave material with m.p. 227.7–228.3°C, $[\alpha]_D$ −16.3°.

Analytical data: calculated for $C_{21}H_{25}NO_{10}$: C, 55.87; H, 5.58; N, 3.10. Found: C, 55.99; H, 5.51; N, 2.96.

NMR data: ^1H, δ 9.89 (s, CHO), 7.81 (d, $J_{m,o}$ 8.7 Hz, H-meta), 7.07 (d, H-ortho), 5.75 (d, $J_{2,NH}$ 8.4 Hz, NH), 5.42 (d, $J_{1,2}$ 8.1 Hz, H-1), 5.43 (dd, $J_{2,3}$ 10.4 Hz, H-3), 5.12 (dd, $J_{3,4}$ 9.2 Hz, H-4), 4.27 (dd, $J_{5,6}$ 5.5, $J_{6,6'}$ 12.2 Hz, H-6), 4.14 (dd, $J_{5,6'}$ 2.5 Hz, H-6′), 4.11 (ddd, H-2), 3.92 (ddd, $J_{4,5}$ 9.4 Hz, H-5), 1.93, 2.03, 2.04, 2.05 (4 s, OAc); ^{13}C, δ 190.8 (CHO), 170.7, 171.4, 169.4 (OAc), 161.7 (O-Ar-ipso), 131.5 (C-meta), 131.3 (C-para), 116.7 (C-ortho), 97.7 (C-1), 72.0 (C-5), 71.7 (C-3), 68.5 (C-4), 61.9 (C-6), 53.9 (C-2), 22.7 (NAc), 20.2, 20.3 (Ac).

p-Formylphenyl 2-acetamido-2-deoxy-β-D-glucopyranoside (**10**)

A suspension of **9** (1.37 g, 3.04 mmol) in methanol (40 ml) was treated with 1 M methanolic sodium methoxide (150 µl). The heterogeneous solution became gradually clear and after several minutes precipitation occurred. More methanol was added (10 ml) and after 35 min TLC ($CHCl_3$/MeOH, 4/1 by vol) indicated complete and quantitative conversion. Ether was then added (50 ml) to ensure complete precipitation of the triol **10**. Filtration and washing of the solid with ether gave pure **10** (965 mg, 98%). Crystallization from ethanol gave material with m.p. 195.0–195.5°C, $[\alpha]_D$ −3.0° (DMSO).

Analytical data: calculated for $C_{15}H_{19}NO_7$: C, 55.50; H, 5.81; N, 4.45. Found: C, 55.38; H, 5.89; N, 4.31.

NMR data: ^1H (DMSO-d_6), δ 9.89 (s, CHO), 7.87 (d, $J_{m,o}$ 8.8 Hz, H-meta), 7.14 (d, H-ortho), 5.14 (d, $J_{1,2}$ 8.4 Hz, H-1), 3.20–3.74 (m, H-2 to H-6), 1.79 (s, NHAc); ^{13}C (DMSO-d_6), δ 192.2 (CHO), 170.6 (NHAc), 162.5 (O-Ar-ipso), 132.2 (C-para), 131.1 (C-meta), 116.9 (C-ortho), 98.7 (C-1), 77.4 (C-5), 74.2 (C-3), 70.5 (C-4), 60.9 (C-6), 55.6 (C-2), 23.2 (NHAc).

p-Formylphenyl 2,3,4,6-tetra-O-acetyl-β-D-glucopyranoside (**12**)

Acetobromoglucose **3** (250 mg, 0.61 mmol), *p*-hydroxybenzaldehyde (150 mg, 1.23 mmol, 2 eq.) and tetrabutylammonium hydrogen sulfate (204 mg, 1 eq.) were dissolved in methylene chloride (2.5 ml). A solution of 1 M NaOH (2.5 ml) was then added. After 40 min, the reaction mixture was processed as above. The residue obtained after evaporation of the organic phase was further purified by column chromatography using ethyl acetate:hexane (1:1 by vol) as eluent to give pure **12** (104 mg, 38%) eluted after the product of elimination, 2,3,4,6-tetra-*O*-acetyl-2-hydroxy-D-glucal (45% yield): m.p. 64.9–66.2°C, $[\alpha]_D$ −33°; lit. [22], m.p. 65–66°C, $[\alpha]_D$ −32°. Compound **12** crystallized from ethanol had m.p. 139.8–141.2°C, $[\alpha]_D$ −16.6°.

Analytical data: calculated for $C_{21}H_{24}O_{11}$: C, 55.75; H, 5.35. Found: C, 55.90; H, 5.47.

NMR data: ^1H, δ 9.90 (s, CHO), 7.83 (d, $J_{m,o}$ 8.7 Hz, H-meta), 7.07 (d, $J_{m,o}$ 8.7 Hz, H-ortho), 5.10 to 5.31 (m, H-1 to H-4), 4.27 (dd, $J_{5,6}$ 5.3 Hz, H-6), 4.15 (dd, $J_{5,6'}$ 2.5 Hz, $J_{6,6'}$ 12.4 Hz, H-6'), 3.90 (ddd, $J_{4,5}$ 9.6 Hz, H-5), 2.04, 2.03, 2.02 (4 s, O-Ac); ^{13}C, δ 190.9 (CHO), 170.6, 170.3, 169.5, 169.4 (Ac), 161.3 (O-Ar-ipso), 131.9 (C-meta and C-para), 116.8 (C-ortho), 97.9 (C-1), 72.4 (C-3), 72.2 (C-5), 70.9 (C-2), 68.0 (C-4), 61.7 (C-6), 20.5, 20.4 (Ac).

p-Formylphenyl β-D-glucopyranoside (**13**)

De-*O*-acetylation of **12** under Zemplén conditions as above (3 h), neutralization with H^+ resin, filtration and evaporation gave a quantitative yield of **13**. Crystallization from methanol/ether gave **13** with m.p. 157.6–159.4°C, $[\alpha]_D$ −48.3° (DMSO).

Analytical data: calculated for $C_{13}H_{16}O_7$: C, 54.93; H, 5.67. Found: C, 54.71; H, 5.69.

NMR data: ^1H (DMSO-d_6), δ 9.89 (s, CHO), 7.87 (s, $J_{m,o}$ 8.8 Hz, H-meta), 7.20 (d, H-ortho), 5.05 (d, $J_{1,2}$ 7.5 Hz, H-1), 3.17–3.70 (m, H-2 to H-6); ^{13}C (DMSO-d_6), δ 191.2 (CHO), 161.9 (O-Ar-ipso), 131.5 (C-meta), 130.3 (C-para), 116.3 (C-ortho), 99.6 (C-1), 77.1 (C-3), 76.4 (C-5), 73.1 (C-2), 69.5 (C-4), 60.5 (C-6).

p-Formylphenyl β-D-lactoside heptaacetate (**15**)

Acetobromolactose **4** (274 mg, 0.39 mmol), *p*-hydroxybenzaldehyde (96 mg, 0.79 mmol, 2 eq.) and tetrabutylammonium hydrogen sulfate (134 mg, 0.40 mmol, 1 eq.) were treated like above in methylene chloride (2 ml) and 1 M NaOH (2 ml). After 45 min at room temperature, the reaction mixture was treated as above. Purification of the residue by column chromatography using ethyl acetate:hexane, 1:1 by vol, containing 0.5% isopropyl alcohol gave pure **15** (131 mg, 45%) followed by peracetylated 2-acetoxy-D-lactal (118 mg, 49%): m.p. 152.7–153.7° (EtOH), $[\alpha]_D$ −19.7°; lit. [22], m.p. 168–170°C, $[\alpha]_D$ −19°. The pure solid **15** which failed recrystallization had m.p. 89.7–91.8°C (slow evaporation of CH_2Cl_2), $[\alpha]_D$ −28.5°.

Analytical data: calculated for $C_{33}H_{40}O_{19}$: C, 52.51; H, 5.44. Found: C, 53.39; H, 5.47.

NMR data: ^1H, δ 9.90 (s, CHO), 7.82 (d, $J_{m,o}$ 8.9 Hz, H-meta), 7.06 (d, H-ortho), 5.34 (dd, $J_{3',4'}$ 3.4 Hz, $J_{4',5'}$ 1.1 Hz, H-4'), 5.28 (ddd, H-5'), 5.11 (dd, $J_{1',2'}$ 7.8 Hz, H-2'), 4.95 (dd, $J_{2',3'}$ 10.4 Hz, H-3'), 4.49 (d, H-1'), 3.84–4.15 (m, remaining), 1.58–2.14 (7s, O-Ac); ^{13}C, δ 190.5 (CHO), 170.2, 170.0, 169.9, 169.8, 169.5, 169.3, 168.9 (C=O), 161.0 (O-Ar-ipso), 131.7 (C-meta, C-para), 116.6 (C-ortho), 101.1 (C-1'), 97.6 (C-1), 76.0 (C-4), 73.0, 72.6, 71.2, 70.9, 70.7, 69.1, 66.6 (ring-C), 61.9, 60.8 (C-6', C-6).

p-Formylphenyl β-D-lactoside (**16**)

The heptaacetate **15** (187 mg, 0.25 mmol) was suspended in methanol (15 ml). Methanolic sodium methoxide (1 M, 20 µl) was then added and the mixture was stirred overnight at room temperature followed by reflux for 10 min to ensure complete de-*O*-acetylation. Ether (15 ml) was added to the cooled solution. After 40 min at 0°C, the product was filtered and dried to give pure **16** (111 mg, quant.), m.p. 244.2–245.9°C (decomp.), $[\alpha]_D$ −11.7° (DMSO).

Analytical data: calculated for $C_{19}H_{26}O_{12}$: C, 51.12; H, 5.87. Found: C, 50.89; H, 6.10.

NMR data: ^1H (^2H_2O), δ 9.83 (s, CHO), 7.97 (d, $J_{m,o}$ 8.9 Hz, H-meta), 7.28 (d, H-ortho), 5.32 (d, $J_{1',2'}$, 7.7 Hz, H-1'), 4.49 (d, $J_{1,2}$ 7.7 Hz, H-1), 3.55–4.03 (m, remaining); ^{13}C (DMSO-d_6), δ 191.2 (CHO), 161.8 (O-Ar-ipso), 131.5 (C-meta), 130.4 (C-para), 116.3 (C-ortho), 103.7 (C-1'), 99.1 (C-1), 79.9, 75.5, 75.0, 74.7, 73.2, 72.8, 70.5, 68.1 (ring-C), 60.4, 60.0 (C-6', C-6).

Direct conjugation of p-formylphenyl glycosides to BSA by reductive amination

Preparation of **8**. Bovine serum albumin (Sigma, Fraction V, 160 mg), aldehyde **7** (255 mg) and sodium cyanoborohydride (49 mg) were incubated at 37°C in 0.2 M sodium phosphate buffer saline (PBS) pH 7.0 (21 ml). The ratio of **7**:NH_2:$NaBH_3CN$ was therefore 4:1:9. Aliquots (2 ml) were withdrawn at time intervals and the reaction mixtures exhaustively dialysed against distilled water. The volumes of the aliquots were then adjusted and the sialic acid contents were determined using the resorcinol method [18]. The protein contents were estimated based on the volume of aliquots withdrawn. The conjugate was isolated by freeze-drying. The experiment was repeated with ratios of **7**:NH_2:$NaBH_3CN$ of 3:1:7 and finally 2:1:2 (Fig. 2).

Preparation of **11**. The above reaction was repeated using the aldehyde **10**. In two sets of experiments, the ratios of **10**:NH_2:$NaBH_3CN$ were 5:1:6 (300 mg **10**, 214 mg BSA, 66 mg $NaBH_3CN$) in 0.2 M PBS pH 7.0 and 2:1:2 (58 mg **10**, 100 mg BSA, 11 mg $NaBH_3CN$), in PBS (10 ml). The

conjugates were isolated by freeze-drying after dialysis against distilled water. In the first set of conjugation, the number of modified lysine residues was determined by amino acid analysis using the phenylisothiocyanate method. In the second set (2:1:2), the UV absorbance was measured at 271 and at 280 nm and standardized using the conjugates **8**.

Preparation of **14** *and* **17**. In each of these two cases, two different reactant ratios were used, aliquots were however not taken at time intervals. For the conjugation of **13** the first molar ratio of **13**:NH_2:$NaBH_3CN$ was 5:1:2 corresponding to 23 mg of **13**, BSA (25 mg) and $NaBH_3CN$ (11.2 mg) in PBS (2.5 ml). In the second experiment, this ratio was 1:1:2 (4.5 mg **13**, 25 mg BSA, 11.2 mg $NaBH_3CN$). The conjugates were processed as before after 90 h at 37°C. The sugar content of **13** in the first conjugate was 47 as measured by the phenol/sulphuric acid method of Dubois *et al.* [19]. The second conjugate has 15 residues of **13** per mole of BSA.

For the lactoside **16** at a similar ratio of 5:1:2 and 1:1:2 the reaction mixtures contained 35.5 mg of **16**, 25 mg BSA, and 11.2 mg $NaBH_3CN$ and 7.1 mg of **16**, 25 mg BSA and 11.2 mg $NaBH_3CN$ respectively in PBS (2.5 ml) at pH 7.0. The content of **16** in the first conjugate was 41/BSA while it was 6/BSA in the second as determined by the colorimetric method described above [19].

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