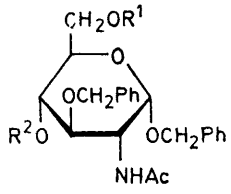
Blood-group Ii-Active Oligosaccharides. Synthesis of a Tetrasaccharide, a β-(1→3) Dimer of *N*-Acetyl-lactosamine

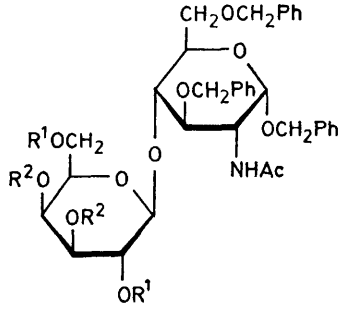
By **Alain Veyrières**, Laboratoire de Chimie Organique Multifonctionnelle, Université de Paris-Sud, 91405 Orsay Cédex, France

Condensation of benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (2) with 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide, followed by de-*O*-acetylation, acetonation, *O*-benzylation, and acidic hydrolysis afforded the 3',4'-diol (5) derived from *N*-acetyl-lactosamine. This compound was selectively glycosylated at the 3'-position by the oxazoline (7) derived from lactosamine to give the protected tetrasaccharide (8) in 52% yield. Removal of the protecting groups gave the free tetrasaccharide β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-D-GlcpNAc (9), a β-(1→3) dimer of *N*-acetyl-lactosamine. This structure has been found in various glycolipids isolated from erythrocyte membranes and could be recognized by several anti-i antisera.

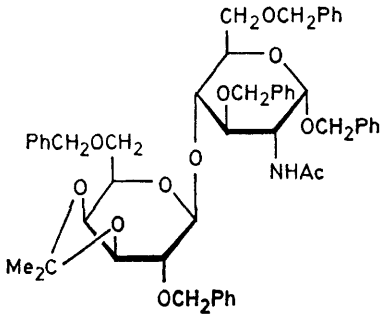
THE human Ii blood-group system shows a great diversity of I and i antigens, as revealed by the heterogeneity of the anti-I and anti-i auto-antibodies of different individuals.[1] The specificity of only two anti-I sera (Ma and Woj) has been recently attributed with some certainty to the presence of the sequence β-D-Galp-(1→4)-β-D-GlcpNAc-(1→6)-D-Gal, which can be considered as the corresponding blood-group antigenic determinant.[2] Strong



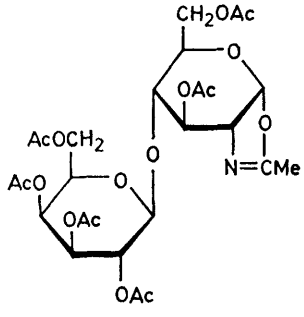
(1) $R^1 = R^2 = H$
(2) $R^1 = CH_2Ph, R^2 = H$



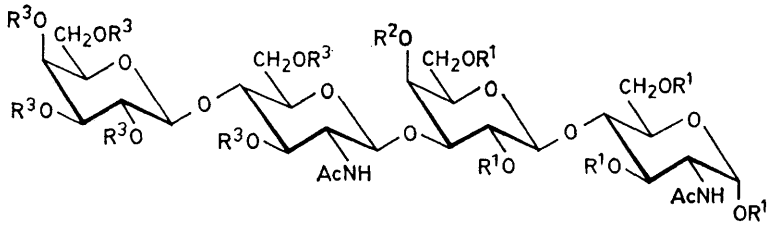
(3) $R^1 = R^2 = Ac$
(5) $R^1 = CH_2Ph, R^2 = H$
(6) $R^1 = CH_2Ph, R^2 = Ac$



(4)



(7)



(8) $R^1 = CH_2Ph, R^2 = H, R^3 = Ac$
(9) $R^1 = R^2 = R^3 = H$

i-activity has been found in a straight-chain ceramide hexasaccharide, ' lacto-*N*-norhexaosylceramide ', β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→1)-Cer, a glycolipid obtained by desialylation of an Ii-active fraction isolated from bovine erythrocyte membranes. Niemann *et al.*[3] postulated that ' the terminal trisaccharide sequence, β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-D-Gal is a necessary but not sufficient requirement for i-activity; the repeat of the penultimate β-D-GlcpNAc-(1→3)-D-Galp disaccharide unit seems to be an additional requirement for the expression of i-specificity '. In this respect, it is of interest that the synthetic trisaccharide, β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-D-Gal, was active as inhibitor of two anti-i sera (McC and Tho), but in 50—100-fold greater amounts than the amounts of lacto-*N*-norhexaosylceramide required to give 50% inhibition of binding.[4]

In 1976, Gardas [5] described a macro-glycolipid containing 22 sugars isolated from human erythrocytes; he proposed for the sugar backbone of this glycolipid a repeating structure, β-D-Galp-(1→4)-[β-D-GlcpNAc-(1→3)-β-D-Galp]$_7$-(1→4)-β-D-Glcp-(1→1)-Cer, which can also be considered as a β-(1→3) heptamer of *N*-acetyl-lactosamine. Such repeating structures seem to be fairly common in various glycolipids designated ' poly-(glycosyl) ceramides ' by Kościelak *et al.*[6] and isolated from human erythrocyte membranes.

These considerations prompted us to develop the synthesis of the tetrasaccharide (9), a β-(1→3) dimer of *N*-acetyl-lactosamine. A similar structure, where the terminal reducing unit *N*-acetylglucosamine has been replaced by glucose, has been found [7] in a ganglioside also isolated from erythrocyte membranes, β-D-Galp-(1→4)-β-D-GlcpNAc-(1→3)-β-D-Galp-(1→4)-β-D-Glcp-(1→1)-Cer, named ' paragloboside '; this glycolipid shows only a weak i-activity in haem-agglutination inhibition tests,[3] and is postulated as a precursor of i-, I-, A-, B-, and H-active glycolipids found in the erythrocyte membrane.[8] Its comparison with the title synthetic tetrasaccharide in inhibition studies with various anti-i sera should contribute to a more precise definition of the antigenic determinant recognized by the various anti-i antibodies.

RESULTS AND DISCUSSION

Our synthetic scheme required the preparation of a derivative of *N*-acetyl-lactosamine suitably protected for a glycosidation at the 3-position of the D-galactose unit. For that purpose, the lactosamine sequence had to be built in a Koenigs–Knorr-type condensation. Jacquinet and Sinaÿ [9] described a high-yield coupling of an orthoester derived from D-galactose to benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (2); the alcohol (2) was obtained by monobenzylation of the diol (1), with moderate yields and difficulties in purification.

We found that the diol (1) could be easily benzylated at the 6-position in 86% yield *via* a tributylstannyl ether prepared according to Ogawa and Matsui; [10] the reaction of benzyl bromide upon the stannylated diol was conducted in the presence of tetrabutylammonium bromide, which strongly accelerates the alkylation of tributylstannyl ethers and dibutylstannylene compounds, as we have shown recently.[11] The selectivity of the reaction was nearly total; column chromatography was nevertheless necessary to remove the tin-containing by-products. While our work was in progress, Petit *et al.*[12] reported another convenient route to compound (2).

The alcohol (2) was glycosidated in 62% yield by 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide [13] in boiling benzene in the presence of mercury(II) cyanide to give the protected disaccharide (3) identical to the product described by Jacquinet and Sinaÿ.[9] This compound was de-*O*-acetylated, then treated with acetone and copper sulphate in the presence of catalytic amounts of concentrated sulphuric acid at room temperature, and *O*-benzylated. The major component of the reaction mixture was isolated in 56% yield and identified as the 3′,4′-*O*-isopropylidene derivative (4), as later shown. A minor compound was also isolated in 8% yield; its ^{1}H n.m.r. spectrum corresponds to an isomeric *O*-isopropylidene derivative, but no further investigation was made on this compound, which is probably the 4′,6′-*O*-isopropylidene isomer, a kinetic product of the acetonation reaction. Acidic hydrolysis of compound (4) led to the desired crystalline 3′,4′-diol (5). Its 250-MHz ^{1}H n.m.r. spectrum showed two doublets exchangeable with deuterium oxide at δ 2.56 and 2.80, corresponding to the 3′- and 4′-OH signals. After *O*-acetylation, the downfield-shifted signals of axial 3′-H and equatorial 4′-H in compound (6) were clearly identified respectively at δ 4.80 as a doublet of doublet ($J_{2',3'}$ 10 Hz and $J_{3',4'}$ 3.5 Hz), and at δ 5.33 as a doublet ($J_{3',4'} = J_{4',5'} = 3.5$ Hz), thus confirming that the major product obtained by acetonation of the de-*O*-acetylated disaccharide was indeed a 3′,4′-*O*-substituted compound.

Condensation of the oxazoline (7) derived from lactosamine [14] with the 3′,4′-diol (5) in the presence of catalytic amounts of toluene-*p*-sulphonic acid gave, after 18 h at 60 °C, 52% of the crystalline tetrasaccharide (8). Only a moderate excess (1.5 equiv.) of oxazoline was used in order to avoid any further glycosidation at the 4-position of the D-galactose unit.[15] De-*O*-acetylation, then hydrogenolysis of the benzyl ether functions, gave the free tetrasaccharide (9), crystallized as a tetrahydrate predominantly in the α-configuration. Its 250-MHz ^{1}H n.m.r. spectrum (solvent D_2O) shows a doublet at δ 5.22 ($J_{1,2}$ 2 Hz) corresponding to the anomeric proton of the terminal reducing *N*-acetyl-D-glucosamine unit in the α-configuration; its intensity allows an estimate of the α : β ratio to be *ca.* 7 : 3. A signal at δ 4.72 ($J_{1'',2''}$ 7.5 Hz) can be attributed to the anomeric proton of the other *N*-acetyl-D-glucosamine residue, thus confirming the β-configuration of the created glycosidic bond; but its intensity corresponds roughly to 1.3 protons; consequently that signal should be also attributed to the anomeric proton of the reducing *N*-acetyl-D-glucosamine

unit in the β-configuration. A similar value (δ 4.74) had been already attributed [2] to the corresponding proton in the disaccharide, β-*N*-acetyl-lactosamine. Finally the two β-(1→4) linked D-galactose residues have their anomeric proton appearing at slightly different values, δ 4.47 and 4.49 (δ 4.49 in *N*-acetyl-lactosamine).

EXPERIMENTAL

Solvents were evaporated under reduced pressure. Ether refers to diethyl ether throughout. Optical rotations were measured at 20 °C with a Roussel-Jouan electronic digital micropolarimeter. N.m.r. spectra were recorded at 250 MHz with a Cameca model STN 250 spectrometer with Fourier-transform unit, with $CDCl_3$ as solvent and tetramethylsilane as internal standard, or with D_2O as solvent and tetramethylsilane (0.2% solution in $CDCl_3$) as external reference. T.l.c. was carried out on plates of silica gel (with fluorescence indicator; layer thickness 0.25 mm, E. Merck, Darmstadt, Germany); ethanolic sulphuric acid (19 : 1, v/v) with charring was used for component detection. Silica gel Merck (70—325 mesh; E. Merck) was used for column chromatography. Paper chromatography was performed on Whatman no. 1 paper. Free sugars were detected with the aniline hydrogenphthalate reagent. Elemental analyses were performed by the Laboratoire Central de Micro-Analyse du C.N.R.S.

Benzyl 2-Acetamido-3,6-di-O-benzyl-2-deoxy-α-D-glucopyranoside (2).—A mixture of benzyl 2-acetamido-3-*O*-benzyl-2-deoxy-α-D-glucopyranoside (1) (0.401 g, 1 mmol) and bis(tributylstannyl) oxide (0.447 g, 0.75 mmol, 1.5 equiv.) in toluene (15 ml) was refluxed for 4 h with continuous removal of water, then concentrated to half-volume, and cooled to 80 °C. Benzyl bromide (0.513 g, 3 mmol) and tetrabutylammonium bromide (0.161 g, 0.5 mmol) were added and the mixture was stirred under nitrogen at 80 °C for 48 h, further addition of benzyl bromide (0.513 g, 3 mmol) and tetrabutylammonium bromide (0.161 g, 0.5 mmol) being made after 24 h. T.l.c. [chloroform–acetone (4 : 1)] showed only traces of starting diol (R_F 0.04) and a major compound (R_F 0.36). The solution was cooled, diluted with chloroform (100 ml), and washed with 10% aqueous potassium hydrogencarbonate solution, then with water; the aqueous washings were re-extracted with chloroform, and the combined extracts were evaporated together with silica gel to form a powder which was applied to a column of silica gel. Elution with chloroform–acetone (9 : 1) gave benzyl 2-acetamido-3,6-di-*O*-benzyl-2-deoxy-α-D-glucopyranoside (2) (0.423 g, 86%), m.p. 143—144 °C (from ethanol–ether) (lit.,[9] m.p. 145—145 5 °C).

Benzyl 2-Acetamido-4-O-(2,3,4,6-tetra-O-acetyl-β-D-galactopyranosyl)-3,6-di-O-benzyl-2-deoxy-α-D-glucopyranoside (3).—A solution of the alcohol (2) (2.95 g, 6 mmol) and mercury(II) cyanide (1.52 g, 6 mmol) in dry benzene (60 ml) was boiled under nitrogen until *ca.* 30 ml of the solvent had distilled. A solution of 2,3,4,6-tetra-*O*-acetyl-α-D-galactopyranosyl bromide (2.47 g, 6 mmol) in dry benzene (30 ml) was rapidly added, and the mixture was refluxed for 30 h, a further addition of bromide (1.24 g, 3 mmol) in dry benzene (30 ml) being made after 6 h. The reaction mixture was cooled to room temperature, diluted with benzene, washed with 10% aqueous potassium iodide solution and water, dried ($MgSO_4$), and evaporated. T.l.c. [ether–ethyl acetate (1 : 1)] showed the presence of some unchanged starting alcohol (R_F 0.55) and a new compound (R_F 0.49). The residue (6.22 g) was chromatographed on silica gel; elution with ether–ethyl acetate (93 : 7) gave the pure protected disaccharide (3) (3.08 g, 62%), m.p. 108 °C (from ether–light petroleum) (lit.,[9] m.p. 110—111 °C).

Benzyl 2-Acetamido-3,6-di-O-benzyl-2-deoxy-4-O-(2,6-di-O-benzyl-3,4-O-isopropylidene-β-D-galactopyranosyl)-α-D-glucopyranoside (4).—A solution of compound (3) (3.08 g, 3.75 mmol) in dry methanol (70 ml) was treated with 1M sodium methoxide in methanol (3.5 ml) at room temperature for 30 min. T.l.c. [chloroform–ethanol (8 : 2)] showed the complete conversion of starting material (R_F 0.73) into the de-*O*-acetylated disaccharide (R_F 0.44). The reaction mixture was neutralized with Amberlite IR 120 (H^+) ion-exchange resin, filtered, and evaporated. The residue was dissolved in dry acetone (150 ml) containing concentrated sulphuric acid (0.22 g); anhydrous copper sulphate (7.2 g) was then added, and the mixture was stirred for 150 min at room temperature. T.l.c. [chloroform–ethanol (9 : 1)] then showed traces of the starting material (R_F 0.17) and a major spot (R_F 0.48). The mixture was filtered, neutralized by stirring with sodium carbonate, filtered again, and evaporated to give a white foam (2.64 g). This material was dissolved in dry *NN*-dimethylformamide (60 ml); barium hydroxide octahydrate (2.3 g), barium oxide (9.0 g), and benzyl bromide (4.3 ml) were successively added, and the mixture was stirred with exclusion of moisture for 3 days at room temperature. T.l.c. [benzene–ethyl acetate (1 : 1)] showed the presence of a major component (R_F 0.35). The reaction mixture was diluted with dichloromethane, filtered, washed with water, and evaporated. The residue was chromatographed on silica gel; elution with toluene–ethyl acetate (2 : 1, then 1 : 1) gave compound (4) as a *syrup* (1.82 g, 56%), $[\alpha]_D$ +73° (*c* 1.01 in chloroform); δ ($CDCl_3$) 1.33 and 1.40 (each 3 H, s, CMe_2), 1.80 (3 H, s, NAc), 4.94 (1 H, d, $J_{1,2}$ 4 Hz, 1-H), 5.31 (1 H, d, *J* 8.5 Hz, NH), and 7.22—7.28 (25 H, 5 Ph) (Found: C, 71.5; H, 6.8; N, 1.4; O, 19.9. $C_{52}H_{59}NO_{11}$ requires C, 71.5; H, 6.8; N, 1.6; O, 20.1%).

Benzyl 2-Acetamido-3,6-di-O-benzyl-2-deoxy-4-O-(2,6-di-O-benzyl-β-D-galactopyranosyl)-α-D-glucopyranoside (5).—A solution of the isopropylidene derivative (4) (1.66 g) in 60% acetic acid was refluxed for 30 min. T.l.c. [chloroform–ethanol (95 : 5)] then indicated complete hydrolysis of the starting material (R_F 0.62) into the diol (5) (R_F 0.44). The solution was cooled, then evaporated, and the residue crystallized from ethanol–light petroleum (1.47 g, 93%), m.p. 167—168 °C, $[\alpha]_D$ +81° (*c* 1.0 in chloroform); δ ($CDCl_3$) 1.80 (3 H, s, NAc), 2.56 (1 H, d, *J* 5 Hz, OH, exchangeable with D_2O), 2.80 (1 H, d, *J* 3 Hz, OH, exchangeable with D_2O), 4.97 (1 H, d, $J_{1,2}$ 4 Hz, 1-H), 5.32 (1 H, d, *J* 8.5 Hz, NH), and 7.29—7.34 (25 H, 5 Ph) (Found: C, 70.5; H, 6.6; N, 1.6; O, 20.9. $C_{49}H_{55}NO_{11}$ requires C, 70.6; H, 6.7; N, 1.7; O, 21.1%).

A portion was *O*-acetylated overnight at room temperature: after removal of the solvents, the residue was dried and examined by ^1H n.m.r. spectroscopy: δ ($CDCl_3$) 1.86 (3 H, s, NAc), 1.96 and 2.00 (each 3 H, s, 2 OAc), 4.80 (1 H, dd, $J_{2',3'}$ 10 Hz, $J_{3',4'}$ 3.5 Hz, 3'-H), 4.96 (1 H, d, $J_{1,2}$ 4 Hz, 1-H), 5.33 (1 H, d, $J_{3',4'} = J_{4',5'}$ 3.5 Hz, 4'-H), 5.38 (1 H, d, *J* 9 Hz, NH), and 7.27—7.30 (25 H, 5 Ph).

O-β-D-Galactopyranosyl-(1→4)-O-2-acetamido-2-deoxy-β-D-glucopyranosyl-(1→3)-O-β-D-galactopyranosyl-(1→4)-2-acetamido-2-deoxy-α-D-glucopyranose (9).—A mixture of the oxazoline (7) (0.93 g, 1.5 mmol), diol (5) (0.83 g, 1 mmol), and anhydrous toluene-*p*-sulphonic acid (50 mg) in dry di-

chloroethane (100 ml) was heated under nitrogen at 60 °C for 18 h. T.l.c. (benzene–ether–methanol, 7 : 7 : 1) then showed a major new compound at R_F 0.30, some unchanged diol (R_F 0.45), and traces of oxazoline (R_F 0.36). The reaction mixture was cooled to room temperature, diluted with dichloromethane, washed with water, and evaporated; the residue was applied to a silica gel column. Elution with toluene–ether–methanol (28 : 28 : 1, then 14 : 14 : 1) gave the pure tetrasaccharide (8) (0.75 g, 52%), which crystallized from ether, m.p. 215—216 °C, $[\alpha]_D$ +39° (*c* 1.05 in chloroform); δ ($CDCl_3$) 1.52 and 1.78 (each 3 H, s, 2 NAc), 1.96, 2.04 and 2.14 (18 H, 6 OAc), 2.69 (1 H, OH, exchangeable with D_2O), 5.11 (1 H, dd, $J_{2'',3''}$ 10.5 Hz, $J_{3'',4''}$ 8 Hz, 3''-H), 5.18 and 5.26 (2 H, d, *J* 9 Hz, 2 NH, move downfield with D_2O), 5.34 (1 H, d, $J_{3''',4'''} = J_{4''',5'''}$ 3 Hz, 4'''-H), and 7.26 (25 H, m, 5 Ph) (Found: C, 62.2; H, 6.4; N, 1.9; O, 29.4. $C_{75}H_{90}N_2O_{27}$ requires C, 62.1; H, 6.3; N, 1.9; O, 29.8%).

A solution of compound (8) (0.40 g) in dry methanol (10 ml) was treated with 1M sodium methoxide in methanol (0.5 ml) for 24 h at room temperature, then neutralized with Amberlite IR 120 (H^+) ion-exchange resin, and evaporated. The residue was dissolved in glacial acetic acid (20 ml) and hydrogenated at room temperature and atmospheric pressure over 10% palladium–charcoal (0.40 g) for 4 days. T.l.c. [propan-2-ol–ethyl acetate–water (3 : 3 : 2)] then indicated complete hydrogenolysis (R_F 0.14). The reaction mixture was evaporated to dryness, without removal of the catalyst; the residue was chromatographed on silica gel using propan-2-ol–ethyl acetate–water (3 : 3 : 1, then 2 : 2 : 1) as eluant The fractions containing the pure tetrasaccharide were combined and evaporated The residue (0.12 g, 53%) crystallized from methanol, m.p. 195—198 °C (decomp.); $[\alpha]_D$ +21° (6 min) to +14° (4 h) (*c* 0.85 in water); paper chromatography: R_{Glc} 0.21 and R_{Lact} 0.43 in ethyl acetate–pyridine–water (2 : 1 : 2) (upper layer); δ (D_2O) 2.02 (6 H, s, 2 NAc), 4.47 and 4.49 (2 H, 2 d, $J_{1',2'} = J_{1''',2'''}$ 7.5 Hz, 1'-H and 1'''-H), 4.72 (1.3 H, d, $J_{1'',2'}$ 7.5 Hz, 1''-H and 1-H_α), and 5.22 (0.7 H, d, $J_{1,2}$ 2 Hz, 1-H_β) (Found: C, 40.9; H, 7.0; N, 3.4; O, 47.5. $C_{28}H_{48}N_2O_{21}\cdot 4H_2O$ requires C, 41.0; H, 6.9; N, 3.4; O, 48.7%).

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