Carbohydrate Research, 142 (1985) 203–211
Elsevier Science Publishers B.V., Amsterdam – Printed in The Netherlands

SYNTHESIS OF *p*-NITROPHENYL β-GLYCOSIDES OF (1→6)-β-D-GALACTOPYRANOSYL-OLIGOSACCHARIDES*

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(Received September 13th, 1984; accepted for publication in revised form, December 1st, 1984)

ABSTRACT

Sequential tritylation, benzoylation, and detritylation of *p*-nitrophenyl β-D-galactopyranoside gave *p*-nitrophenyl 2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside (**2**). Reaction of **2** with 2,3,4,6-tetra-*O*-benzoyl-α-D-galactopyranosyl bromide gave *p*-nitrophenyl *O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl)-(1→6)-2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside (**4**) in 94% yield. Deprotection with sodium methoxide then gave the crystalline *p*-nitrophenyl *O*-(β-D-galactopyranosyl)-(1→6)-β-D-galactopyranoside (**5**). Condensation of **2** with 2,3,4-tri-*O*-benzoyl-6-*O*-bromoacetyl-α-D-galactopyranosyl bromide (**3**) readily yielded the protected disaccharide *p*-nitrophenyl *O*-(2,3,4-tri-*O*-benzoyl-6-*O*-bromoacetyl-β-D-galactopyranosyl)-(1→6)-2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside (**6**) from which the bromoacetyl groups could be selectively removed. Condensation of the resulting material with tetra-*O*-benzoyl-α-D-galactopyranosyl bromide then yielded *p*-nitrophenyl *O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-benzoyl-β-D-galactopyranosyl)-(1→6)-2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside, (**8**), which was converted into the crystalline trisaccharide *p*-nitrophenyl *O*-(β-D-galactopyranosyl)-(1→6)-*O*-β-D-galactopyranosyl)-(1→6)-β-D-galactopyranoside (**9**) by treatment with sodium methoxide. Preliminary experiments on the interaction of *p*-(bromoacetamido)phenyl and *p*-isothiocyanatophenyl glycoside derivatives of some of these galacto-saccharides with monoclonal anti-(1→6)-β-D-galactopyranan antibodies have been conducted.

INTRODUCTION

The combining site of a (1→6)-β-D-galactopyranan-binding monoclonal antibody has recently been mapped through the use of deoxyfluoro galactosyl derivatives as ligands in binding studies[1]. Binding most probably involves hydrogen-[1] as

*Part I of the series Affinity labels for anti-(1→6)-β-D-galactopyranan antibodies.
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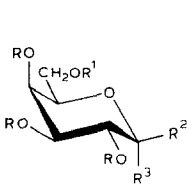
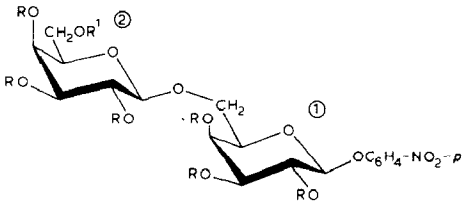
well as hydrophobic-bonding, as proposed by us[2]. Subsequent thermodynamic mapping[3] of the binding of two monoclonal antibodies to their respective saccharide ligands agreed with these proposals. The use of specifically substituted deoxyfluoro sugars as ligands[1,4] made possible great strides in evaluating the antigen–antibody interaction, but it is clear that the ultimate visualization of this interaction would be to obtain X-ray diffraction patterns of the immunoglobulin while ligand occupies the antibody combining-site. Efforts to do so in the case of the immunoglobulin studied by us have not met with success[5] and we have therefore turned our attention to affinity labelling as a possible way to increase our insight. We report here the preparation of two galacto-oligosaccharides bearing a glycosidic *p*-nitrophenyl group. The nitro group may be readily converted, *via* an amino function, into a number of groups, such as isothiocyanate, diazo, or *N*-bromoacetate, capable of reacting with nucleophilic amino acid residues of an antibody combining-site

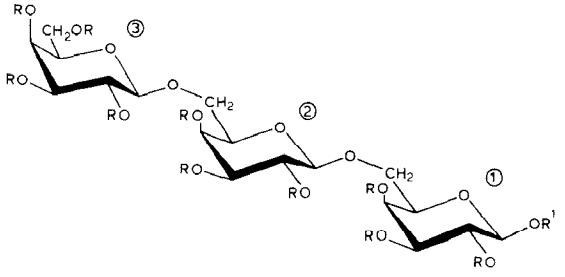
RESULTS AND DISCUSSION



	R	R^1	R^2	R^3
1	Bz	Ph_3C	OC_6H_4-NO_2-*p*	H
2	Bz	H	OC_6H_4-NO_2-*p*	H
3	Bz	$OCCH_2Br$	H	Br



	R	R^1
4	Bz	Bz
5	H	H
6	Bz	$OCCH_2Br$
7	Bz	H



8 R = Bz, R^1 = $C_6H_4NO_2$-*p*
9 R = H, R^1 = $C_6H_4NO_2$-*p*
13 R = H, R^1 = C_6H_4NCS-*p*

The starting unit for synthesis of the di- (**5**) as well as the tri-saccharide (**9**) was **2**. *p*-Nitrophenyl β-D-galactopyranoside was treated with chlorotriphenylmethane in pyridine and the resulting 6-*O*-trityl derivative was benzoylated *in situ*. The product (**1**) was directly detritylated by using 90% acetic acid in water to yield the partially protected derivative **2** in 82% yield. For synthesis of the digalactoside,

the nucleophile **2** was treated with tetra-*O*-benzoyl-α-D-galactopyranosyl bromide by using the silver triflate–2,4,6-collidine complex in anhydrous toluene–nitromethane[6] as a promoter. After processing and chromatography, the crystalline, fully protected disaccharide derivative **4** was isolated in 94% yield. Compound **4** was then debenzoylated by sodium methoxide in methanol to give **5** in 86% yield. The ^{1}H-n.m.r. spectrum of **5** shows *inter alia* a one-proton doublet at δ 5.13 having a coupling constant of 7.2 Hz assigned to H-1 of the galactosyl residue-1, and another one at δ 4.38 with a coupling constant of 7.1 Hz, assigned to H-1 of the galactosyl group-2. The ^{13}C-n.m.r. spectrum of **5** showed, *inter alia*, resonances at δ 103.44 (C-1, β-galactosyl group-2) and 100.14 (C-1, β-galactosyl residue-1); the signal at δ 69.03 was assigned to C-6 of the galactosyl residue-1, substantially downfield of the resonance for C-6 of the galactosyl group-2 at δ 61.06. The latter value is typical for an unsubstituted 6-position in a hexopyranose. These data, together with the magnitude of the specific optical rotation of $[\alpha]^{23}_{578}$ −67°, thus demonstrate that **5** has the designated structure.

In the synthesis of the trigalactoside **9**, the nucleophile **2** was first condensed with 2,3,4-tri-*O*-benzoyl-6-*O*-bromoacetyl α-D-galactopyranosyl bromide[7] (**3**), again using the silver triflate–2,4,6-collidine complex[6] as a promoter. After processing and chromatography, the intermediate, fully protected, derivative **6** was obtained in 72% yield. Its 6-*O*-bromoacetyl group was removed by treatment with thiourea in dichloromethane–methanol to afford the disaccharidic nucleophile **7** in 79% yield. This compound was then condensed with tetra-*O*-benzoyl-α-D-galactopyranosyl bromide, again using the silver triflate–2,4,6-collidine complex[6], to give, after purification, the fully protected trisaccharide derivative **8** in 86% yield. Compound **8** was then debenzoylated with sodium methoxide in methanol–ethyl acetate (the latter necessary to improve the solubility of **8**; transesterification of the ethyl acetate did not interfere with the debenzoylation of **8**) to give the trigalactoside **9** in 93% yield. In the ^{1}H-n.m.r. spectrum, **9**, showed one-proton doublets at δ 5.12, coupling constant 7 Hz (H-1, Gal-1), at δ 4.40 and 4.36 with coupling constants of 7.4 Hz and 7.7 Hz, respectively, assigned to the anomeric protons of Gal-2 and Gal-3. The ^{13}C-n.m.r. spectrum of **9** showed signals at δ 103.54 assigned to C-1 of both Gal-2 and Gal-3. At δ 100.12 a signal was observed for C-1 of the galactoside residue-1. The signal at δ 69.32 was assigned to C-6 of both the galactoside residue-1 and the galactosyl residue-2, and at δ 61.09 a resonance was observed for C-6 of the galactosyl group-3. These data, and an optical rotation of $[\alpha]^{23}_{578}$ −46°, support the structure assigned to **9**.

We have shown that *p*-nitrophenyl β-D-galactopyranoside may be readily converted into *p*-bromoacetamidophenyl β-D-galactopyranoside (**10**). This compound was shown to react smoothly with a nucleophile such as *p*-thiocresol in an exploratory experiment. Thus these compounds appear to be suitable for reaction with nucleophilic amino acid residues such as may occur in the combining site of monoclonal antibodies to be studied. It was still necessary to show that a representative compound of this kind would show *affinity* for a monoclonal anti-(1→6)-

β-D-galactopyranan antibody. Thus *p*-acetamidophenyl β-D-galactopyranoside (**11**) was prepared, and its affinity constant, K_a, was measured with monoclonal anti-galactan IgA J539 Fab', using ligand-induced tryptophanyl fluorescence change[8] (the *p-bromo*acetamido derivative would be unsuitable for measurement because of the possibility of actual covalent interaction between ligand and antibody during the titration). This showed the reasonably high degree of binding of K_a 0.5×10^4. Secondly, Ouchterlony double-diffusion[9] showed that compound **10** was capable of inhibiting the precipitin reaction between whole IgA J539 (ascites) and lung galactan[10].

In addition, *p*-isothiocyanatophenyl β-D-galactopyranoside[11] (**12**) and *p*-isothiocyanatophenyl *O*-(β-D-galactopyranosyl)-(1→6)-β-D-galactopyranoside (**13**) were prepared and they were shown to block the combining sites of monoclonal IgA's X24 and J539, respectively[12,13].

EXPERIMENTAL

General. — Methods were essentially as reported before[14]. For ^{13}C-n.m.r. spectroscopy in D_2O, methanol (δ 49.0) was used as an internal reference, and for ^{1}H-n.m.r. spectroscopy the HDO peak (δ 4.78 at 30°) was used as the internal reference.

p-*Nitrophenyl 2,3,4-tri*-O-*benzoyl*-β-D-*galactopyranoside* (**2**). — *p*-Nitrophenyl β-D-galactopyranoside (2 g, 6.64 mmol) and chlorotriphenylmethane (3.5 g, 12.5 mmol) were dissolved in anhydrous pyridine (40 mL) and the mixture was stirred for three days at room temperature. Examination by t.l.c. (9:1 chloroform–methanol) showed the presence of only one product at R_F 0.25, and no remaining starting-material. The mixture was cooled, with continued stirring in an ice–water bath, and benzoyl chloride (3 mL, 25.7 mmol) was added dropwise. The bath was removed, and the mixture was stirred overnight at room temperature. T.l.c. revealed the presence of only one major product, at R_F ~0.48 (16:1 toluene–ethyl acetate). Water (1 mL) was added to decompose reagents. After 30 min, the mixture was diluted with toluene (200 mL), washed with water (2 × 100 mL), saturated aqueous $NaHCO_3$ (2 × 100 mL), and again water (100 mL), and dried (Na_2SO_4). The mixture was filtered and the filtrate evaporated to a thick syrup. Residual pyridine was removed by repeated evaporation of toluene from the residue. In a separate experiment, the intermediate tritylated, benzoylated derivative **1** was isolated and crystallized, m.p. 210–211°. The residue was dissolved in 90% acetic acid in water (100 mL) and heated on a steam bath for 30 min. After cooling and evaporation, remaining acetic acid was removed by evaporation of toluene from the residue. Examination by t.l.c. (4:1 toluene–ethyl acetate) showed one major product, R_F 0.21. Chromatography on a column of silica gel with the same solvents gave 3.35 g (82%) of pure **2** as an amorphous solid, $[\alpha]_{578}$ +173° (*c* 1.26, $CHCl_3$); ^{1}H-n.m.r. (220 MHz, $CDCl_3$ +20% CD_3OD): δ 8.25–7.05 (m, 19 H, aromatic H), 6.34 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 Hz, H-2), 6.29 (d, 1 H, $J_{3,4}$ 3.5, $J_{4,5}$ <1

Hz, H-4), 5.84 (dd, 1 H, $J_{2,3}$ 10, $J_{3,4}$ 3.5 Hz, H-3), 5.66 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.41 (dd, 1 H, $J_{5,6} = J_{6,6a}$ 6.5 Hz, H-6), and 3.95–3.73 (m, 2 H, H-5 and H-6a); ^{13}C-n.m.r. (75 MHz, $CDCl_3$ + 20% CD_3OD) showed *inter alia* δ 99.02 (C-1), 75.07 (C-5), 72.06 (C-3), 69.80 (C-2), 68.51 (C-4), and 60.41 (C-6).

p-*Nitrophenyl* O-*(2,3,4,6-tetra-*O*-benzoyl-β-*D*-galactopyranosyl)-(1→6)-2,3,4-tri-*O*-benzoyl-β-*D*-galactopyranoside* (**4**). — The nucleophile **2** (1.27 g, 2 mmol) and tetra-*O*-benzoyl-α-D-galactopyranosyl bromide (1.45 g, 2.2 mmol) were dissolved in anhydrous toluene–nitromethane (1:1, 10 mL), and the solution was cooled to −30° under dry argon. A solution of silver triflate (0.57 g, 2.2 mmol) and *sym*-collidine (225 μL, 1.7 mmol) in dry toluene–nitromethane (5 mL) was added dropwise, with stirring, over a period of 5 min. After 15 min at −30°, t.l.c. (8:1 toluene–ethyl acetate) showed the presence of one major product (R_F 0.43), and no remaining starting-material. The mixture was made neutral by the addition of pyridine (0.5 mL) and filtered through Celite. The filtrate was diluted with toluene (100 mL) and washed with water (50 mL), 0.5M $Na_2S_2O_3$ (2 × 25 mL) and, again, water, dried (Na_2SO_4) and concentrated. Purification on a column of silica gel (8:1 toluene–ethyl acetate) gave pure **4** (2.25 g, 94%) crystalline. After recrystallization from ethanol it had m.p. 147–149° and $[\alpha]_{578}^{23}$ +117° (*c* 2.22, $CHCl_3$); ^{1}H-n.m.r. (220 MHz, $CDCl_3$): δ 8.20–6.95 (m, 39 H, aromatic H), 6.07 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 Hz, H-2), 6.00 (d, 2 H, $J_{3,4}$ 3.5, $J_{4,5}$ 1 Hz, H-4 and H-4'), 5.89 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 Hz, H-2'), 5.68 (dd, 1 H, $J_{2,3}$ 10, $J_{3,4}$ 3.5 Hz, H-3'), 5.41 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.95 (d, 1 H, $J_{1,2}$ 8 Hz, H-1'), 4.66–4.20 (m, 5 H, H-5', 2 × H-6, 2 × H-6'), and 3.98 (dd, 1 H, $J_{5,6}$ 8, $J_{5,6a}$ 10 Hz, H-5); ^{13}C-n.m.r. (75 MHz, $CDCl_3$) showed *inter alia*: δ 101.23 (C-1'), 99.12 (C-1), 73.71 (C-5), 71.80, 71.63 and 71.41 (C-3, C-3' and C-5'), 69.64 and 69.35 (C-2 and C-2'), 68.40 (C-4), 68.20 (C-6 and C-4'), and 62.04 (C-6').

Anal. Calc. for $C_{67}H_{53}NO_{20}$: C, 67.50; H, 4.48; N, 1.18. Found: C, 67.30; H, 4.31; N, 1.35.

p-*Nitrophenyl* O-β-D-*galactopyranosyl-(1→6)-β-*D*-galactopyranoside* (**5**). — The protected disaccharide derivative **4** (2.06 g, 1.73 mmol) was suspended in methanol (50 mL) and sodium methoxide in methanol (0.2M, 3 mL) was added. The mixture was heated under reflux for 15 min on a steam bath, whereupon a clear solution was obtained. After an additional 15 h at room temperature, sodium ions were removed by treatment with Amberlite IR-120 (H^+) resin, and the solution was evaporated to a crystalline residue. Recrystallization from a small amount of methanol yielded pure **5** (0.57 g, 71%). Chromatography of the mother liquors on a column of silica gel (6:2:1 ethyl acetate–propanol–water) afforded additional **5** (0.12 g), bringing the total yield to 86%. An analytical sample had m.p. 215–218°, $[\alpha]_{578}^{23}$ −67° (*c* 1.47, water); ^{1}H-n.m.r. (300 MHz, D_2O): δ 8.15 and 7.35 (both d, 2 H each, AB spectrum, $J_{H,H}$ 9 Hz, *p*-nitro-C_6H_4O-group), 5.13 (d, 1 H, $J_{1,2}$ 7.2 Hz, H-1), 4.38 (d, 1 H, $J_{1,2}$ 7.1 Hz, H-1'), and 4.12 (dd, 1 H, $J_{2,3}$ 7.2, $J_{3,4}$ 3.8 Hz, H-3); ^{13}C-n.m.r. (25.05 MHz, D_2O): δ 103.44 (C-1'), 100.13 (C-1), 75.27 (C-5'), 74.59 (C-5), 72.88 (C-3'), 72.44 (C-3), 70.93 (C-2'), 70.44 (C-2), 69.03 (C-6), 68.74 and 68.64 (C-4,4'), and 61.06 (C-6').

Anal. Calc. for $C_{18}H_{25}NO_{13} \cdot 0.5\ H_2O$: C, 45.76; H, 5.55; N, 2.96. Found: C, 45.79; H, 5.33; N, 3.01.

p-*Nitrophenyl* O-*(2,3,4-tri*-O-*benzoyl-6*-O-*bromoacetyl-β*-D-*galactopyranosyl)-(1→6)-2,3,4-tri*-O-*benzoyl-β*-D-*galactopyranoside* (**6**). — A solution of the nucleophile **2** (1.64 g, 2.67 mmol) and the 6-*O*-bromoacetyl benzobromogalactose bromide **3** (1.99 g, 2.94 mmol) in anhydrous toluene–nitromethane (1:1, 15 mL) was stirred and cooled to −30° under dry argon. A solution of silver triflate (0.77 g, 2.94 mmol) and *sym*-collidine (300 μL, 2.27 mmol) in toluene–nitromethane (10 mL) was added dropwise over a period of 5 min. After stirring for 15 min more at −30°, examination by t.l.c. (8:1 toluene–ethyl acetate) showed a major product at R_F 0.38. After processing and chromatography (see the preparation of **4** for details), pure **6** (2.34 g, 72%) was obtained crystalline. An analytical sample had m.p. 219–222° (sintering at 130–135°), $[\alpha]_{578}^{23}$ +145° (*c* 1.3, $CHCl_3$); ^{1}H-n.m.r. (220 MHz, $CDCl_3$): δ 8.18–6.95 (m, 34 H, aromatic H), 6.06 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 Hz, H-2), 6.02 (d, 1 H, $J_{3,4}$ 3, $J_{4,5}$ 1 Hz, H-4), 5.89 (d, 1 H, $J_{3,4}$ 3, $J_{4,5}$ <1 Hz, H-4′), 5.84 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 Hz, H-2′), 5.70 (dd, 1 H, $J_{2,3}$ 10, $J_{3,4}$ 3 Hz, H-3), 5.52 (dd, 1 H, $J_{2,3}$ 10, $J_{3,4}$ 3 Hz, H-3′), 5.43 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 4.89 (d, 1 H, $J_{1,2}$ 8 Hz, H-1′), 4.52 (m, 1 H, H-6), 4.43–4.16 (m, 4 H, H-6a, H-5, and 2 × H-6′), 3.93 (dd, 1 H, $J_{5,6}$ 8, $J_{5,6a}$ 10 Hz, H-5), and 3.78 (s, 2 H, bromoacetyl-CH_2); ^{13}C-n.m.r. (25.05 MHz, $CDCl_3$): δ 101.13 (C-1′), 99.18 (C-1), 73.79 (C-5), 71.74 (C-5′), 71.74 (C-3 and C-3′), 69.45 and 69.26 (C-2 and C-2′), 68.42 (C-4), 68.04 (C-6 and C-4′), and 63.46 (C-6′).

Anal. Calc. for $C_{62}H_{50}BrNO_{20}$: C, 61.59; H, 4.17; N, 1.16. Found: C, 61.89; H, 3.92; N, 1.45.

p-*Nitrophenyl* O-*(2,3,4-tri*-O-*benzoyl-β*-D-*galactopyranosyl)-(1→6)-2,3,4-tri*-O-*benzoyl-β*-D-*galactopyranoside* (**7**). — To a solution of compound **6** (2.23 g, 1.93 mmol) in dichloromethane (20 mL) was added a solution of thiourea (0.45 g, 5.8 mmol) in methanol (35 mL). After 15 min at room temperature, t.l.c. (4:1 toluene–ethyl acetate) showed a new, major, product having R_F 0.28, and moving slower than the starting material (R_F 0.60, of which only traces remained), as well as some strongly u.v.-absorbing (but not charring) material remaining at the origin. The solution was diluted with toluene (250 mL), washed with water (3× 100 mL), dried (Na_2SO_4) and evaporated to a crystalline residue. Recrystallization from methanol gave pure **7** (1.65 g, 79%). An analytical sample had m.p. 154–156°, $[\alpha]_{578}^{23}$ +180.5° (*c* 1.39, $CHCl_3$); ^{1}H-n.m.r. (220 MHz, $CDCl_3$ + 20% CD_3OD): δ 8.23–7.00 (m, 34 H, aromatic H), 6.11 and 5.98 (d and dd, 2 H, overlapping signals for H-2 and H-4), 5.92 (d, 1 H, $J_{3,4}$ 3, $J_{4,5}$ <1 Hz, H-4′), 5.84 (dd, 1 H, $J_{1,2}$ 7.5, $J_{2,3}$ 10 Hz, H-2′), 5.75 (dd, 1 H, $J_{2,3}$ 10.5, $J_{3,4}$ 3 Hz, H-3), 5.57 (d and dd, 2 H, coincident signals, d has $J_{1,2}$ 8 Hz, H-1, and dd has $J_{2,3}$ 10, $J_{3,4}$ 3 Hz, H-3′), 4.95 (d, 1 H, $J_{1,2}$ 7.5 Hz, H-1′), 4.63 (dd, 1 H, $J_{5,6}$ 8, $J_{6,6a}$ 3.5 Hz, H-6), 4.27 (dd, 1 H, $J_{5,6a}$ 10.5, $J_{6,6a}$ 3.5 Hz, H-6a), 4.09 (dd, 1 H, $J_{5,6}$ 6, $J_{5,6a}$ 6 Hz, H-5′), 3.98 (dd, 1 H, $J_{5,6}$ 8, $J_{5,6a}$ 10.5 Hz, H-5), and 3.82–3.59 (m, 2 H, 2 × H-6′); ^{13}C-n.m.r. ($CDCl_3$ + 20% CD_3OD): δ 101.37 (C-1′), 99.13 (C-1), 74.62 (C-5′), 73.74 (C-5), 72.57 and 71.89 (C-3 and

C-3'), 70.23 and 69.69 (C-2 and C-2'), 68.92 (C-4 and C-4'), 68.57 (C-6), and 60.58 (C-6').

Anal. Calc. for $C_{60}H_{49}NO_{19}$: C, 66.23; H, 4.54; N, 1.29. Found: C, 66.16; H, 4.49; N, 1.57.

p-*Nitrophenyl* O-*(2,3,4,6-tetra*-O-*benzoyl*-β-D-*galactopyranosyl)*-*(1→6)*-O-*(2,3,4-tri*-O-*benzoyl*-β-D-*galactopyranosyl)*-*(1→6)*-*2,3,4-tri*-O-*benzoyl*-β-D-*galactopyranoside* (**8**). — The partially protected disaccharide **7** (1.56 g, 1.43 mmol) and tetra-*O*-benzoyl-α-D-galactopyranosyl bromide (1.04 g, 1.58 mmol) were condensed at −25°, using silver triflate (0.41 g, 1.58 mmol) and *sym*-collidine (160 μL, 1.22 mmol) exactly as described for the preparation of **4**. Processing and purification as for **4** gave pure **8** (2.04 g, 86%), which crystallized on being kept. An analytical sample had m.p. 147–151°, $[\alpha]_{578}^{23}$ +111.5° (*c* 1.86, $CHCl_3$); ^{1}H-n.m.r. (220 MHz, $CDCl_3$): δ 8.18–6.98 (m, 54 H, aromatic H), 6.13 (dd, 1 H, $J_{1,2}$ 8, $J_{2,3}$ 10 MHz, H-2), 6.05 (d, 1 H, $J_{3,4}$ 3.5, $J_{4,5}$ 1 Hz, H-4), 5.95 (d, 1 H, $J_{3,4}$ 3.5, $J_{4,5}$ 1 Hz, H-4''), 5.90 (d, 1 H, $J_{3,4}$ 3, $J_{4,5}$ 1 Hz, H-4'), 5.84–5.68 (m, 3 H, H-3, H-2', and H-2''), 5.60 (dd, 1 H, $J_{2,3}$ 10.5, $J_{3,4}$ 3.5 Hz, H-3''), 5.49 (d, 1 H, $J_{1,2}$ 8 Hz, H-1), 5.36 (dd, 1 H, $J_{2,3}$ 10, $J_{3,4}$ 3 Hz, H-3'), 4.78 (d, 2 H, coincident signals, $J_{1,2}$ 7.5 Hz for both, H-1' and H-1''), 4.58 (dd, 1 H, $J_{5,6}$ 5, $J_{5,6a}$ 10 Hz, H-6''), 4.52 (dd, 1 H, $J_{5,6}$ 7, $J_{6,6a}$ 3.5 Hz, H-6a), 4.36–4.09 (m, 4 H, H-5', H-5'', H-6a and H-6'), 4.00 (dd, 1 H, $J_{5,6a}$ 5, $J_{6,6a}$ 10.5 Hz, H-6a''), 3.93 (dd, 1 H, $J_{5,6a}$ 2.5, $J_{6,6a}$ 10.5 Hz, H-6a''), 3.93 (dd, 1 H, $J_{5,6a}$ 2.5, $J_{6,6a}$ 10.5 Hz, H-6a'), and 3.83 (dd, 1 H, $J_{5,6}$ 7, $J_{5,6a}$ 10 Hz, H-5); ^{13}C-n.m.r. (25.05 MHz, $CDCl_3$): δ 101.62 (C-1''), 101.23 (C-1'), 99.18 (C-1), 73.40 (C-5), 73.01 (C-5'), 71.84 and 71.50 (C-3, C-3', C-3'' and C-5''), 69.89, 69.69, and 69.40 (C-2, C-2' and C-2''), 68.38 and 68.18 (C-4, C-4', C-4'', C-6, and C-6'), 61.95 (C-6'').

Anal. Calc. for $C_{94}H_{75}NO_{28}$: C, 67.74; H, 4.54; N, 0.84. Found: C, 67.89; H, 4.74; N, 0.92.

p-*Nitrophenyl* O-β-D-*galactopyranosyl*-*(1→6)*-O-*(*β-D-*galactopyranosyl)*-*(1→6)*-β-D-*galactopyranoside* (**9**). — Compound **8** (1.89 g, 1.13 mmol) was dissolved in a mixture of methanol (50 mL) and ethyl acetate (20 mL), and sodium methoxide in methanol (0.2M, 3 mL) was added. After stirring overnight, part of the product had precipitated and was dissolved by the addition of water (70 mL). Sodium ions were removed by treatment with Amberlite IR-120 (H^+) resin, and the solution was evaporated to a crystalline residue. Recrystallization from methanol gave pure **9** (0.66 g, 93%), m.p. 189–193°, $[\alpha]_{578}^{23}$ −46° (*c* 1.22, water); ^{1}H-n.m.r. (300 MHz, D_2O): δ 8.23 and 7.27 (both d, 2 H each, AB-spectrum, $J_{H,H}$ 9 Hz, *p*-nitro-C_6H_4O-group), 5.17 (d, 1 H, $J_{1,2}$ 7 Hz, H-1), 4.40 (d, 1 H, $J_{1,2}$ 7.4 Hz, H-1' or H-1''), 4.36 (d, 1 H, $J_{1,2}$ 7.7 Hz, H-1'' or H-1'), and 4.16 (dd, 1 H, $J_{2,3}$ 7.8, $J_{3,4}$ 4.1 Hz, H-3); ^{13}C-n.m.r. (25.05 MHz, D_2O): δ 103.54 (C-1',1''), 100.12 (C-1), 75.22 (C-5''), 74.59 (C-5), 73.86 (C-5'), 72.88 (C-3'), 72.74 (C-3''), 72.44 (C-1), 70.88 (C-2',2''), 70.49 (C-2), 69.32 (C-6,6'), 68.69 (C-4,4',4''), and 61.09 (C-6'').

Anal. Calc. for $C_{24}H_{35}NO_{18} \cdot H_2O$: C, 44.79; H, 5.80; N, 2.18. Found: C, 44.79; H, 5.91; N, 2.16.

p-*(Bromoacetamido)phenyl* β-D-*galactopyranoside* (**10**). — *p*-Aminophenyl

β-D-galactopyranoside (500 mg, 1.63 mmol; Sigma Chemical Co.) was suspended in dry 1,4-dioxane (6 mL) and solid bromoacetic anhydride (523 mg, 2.03 mmol) was added while the mixture was cooled in ice. The ice bath was removed, and more 1,4-dioxane (20 mL) was added. The mixture was stirred for 45 min, filtered, and the residue was washed with ether. The residue (589 mg, single spot on t.l.c. in 6:5 ethyl acetate–ethanol, traveling faster than the starting material) was recrystallized from methanol–ether to give 129 mg of **10**, m.p. 181–182°.

Anal. Calc. for $C_{14}H_{18}BrNO_7$: Br, 20.46. Found: Br, 20.65.

p-*Acetamidophenyl β-D-galactopyranoside* (**11**). — *p*-Aminophenyl β-D-galactopyranoside (0.53 g) was acetylated conventionally in pyridine (7 mL) with acetic anhydride (5 mL). *O*-Deacetylation with 0.2M sodium methoxide followed by deionization (Amberlite IR-120, H^+) and recrystallization of the resulting product from methanol gave **11** (294 mg), m.p. 215–218°; c.i.–m.s. (NH_3) *m/z* 331 (M + 18).

Anal. Calc. for $C_{14}H_{19}NO_7 \cdot H_2O$: C, 50.75; H, 6.34; N, 4.23. Found: C, 51.03; H, 6.56; N, 4.12.

p-*Isothiocyanatophenyl β-D-galactopyranoside* (**12**). — This compound was prepared from *p*-aminophenyl β-D-galactopyranoside essentially as described by Buss and Goldstein[11], and had m.p. 210° (lit. 210–212°).

Anal. Calc. for $C_{13}H_{15}NO_6S$: C, 49.84; H, 4.97; N, 4.47; S, 10.22. Found: C, 49.56; H, 5.17; N, 4.43; S, 10.63.

The i.r. spectrum of **12** showed the characteristic N=C=S absorption at 2080 cm^{-1}.

p-*Isothiocyanatophenyl O-β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside* (**13**). — Compound **9** (100 mg) was hydrogenated in 3:1 ethanol–water, over 10% palladium-on-charcoal, and the resulting amine was purified by chromatography on silica gel (1:1 chloroform–methanol). The product was directly treated with thiophosgene as described[11] and the resulting material was crystallized from ethanol to give **13**, m.p. 210–215° (dec.), $[\alpha]_{589}^{20}$ −42° (*c* 0.1, methanol). The i.r. spectrum of this material showed the characteristic N=C=S absorption at 2050 cm^{-1}.

Binding studies of certain ligands with monoclonal immunoglobulins. — After it had been shown that **10** reacted essentially to completion in 6 h with *p*-thiocresol to yield a faster-moving product (silica gel t.l.c., 6:2:1 ethyl acetate–propanol–water), presumably *p*-[*S*-(4-methylphenyl)thioglycolyl]amidophenyl β-D-galactopyranoside, the binding affinity of **11** was measured as described before[8] with monoclonal J539 Fab′, and a value of K_a 0.5 × 10^4 was found. When a solution of mammalian lung galactan [a branched polysaccharide having side chains of β-(1→6)-linked D-galactopyuranosyl residues[10]] at 0.2% was run in agar gels in phosphate-buffered saline (pH 7.4) *vs.* whole monoclonal IgA J539 (ascites), heavy precipitin lines were obtained which could be completely inhibited by prior incubation of the gel with *p*-(bromoacetamido)phenyl β-D-galactopyranoside (**10**). Finally, the following experiments were conducted: Whole monoclonal antigalactan

IgA X24 (affinity purified[13], 100 μL, of 1.3 absorbance$_{280\ nm}$/mL) and 400 μL of an aqueous solution of *p*-isothiocyanatophenyl β-D-galactopyranoside (1 mg/mL) were combined and the pH of the solution was adjusted to 9. The mixture was kept for 4 h at room temperature and the pH was adjusted to neutrality. This solution was added to the top of a column of Biogel P-10 (1.6 × 55 cm) that was eluted with 0.01M phosphate-buffered saline (PBS) pH 7.4. The putatively cold-labeled monoclonal IgA, separated from excess labeling agent, was pooled, and had an absorbance$_{280\ nm}$ of 0.265 unit. Addition of methyl *O*-β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside[15] (10 μL, 8.8mM) failed to change the tryptophanyl fluorescence. Addition of the same amount of methyl *O*-β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside to unreacted whole IgA X24 caused a ligand-induced change in the tryptophanyl fluorescence of 15%, thus indicating that the cold-labeled IgA exhibited a blocked combining-site.

In a similar experiment, whole, affinity purified[13], IgA J539 was treated with **13**, and the resulting antibody was purified by dialysis against PBS of pH 7.4. This protein showed sharply diminished, ligand-induced tryptophanyl fluorescence-change when methyl *O*-β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside was added, again indicating that the combining site was blocked.

REFERENCES

1 C. P. J. GLAUDEMANS, P. KOVÁČ, AND K. RASMUSSEN, *Biochemistry*, 23 (1984) 6732–6736.
2 M. K. DAS, E. ZISSIS, AND C. P. J. GLAUDEMANS, *Carbohydr. Res.*, 73 (1979) 235–244.
3 A. K. BHATTACHARJEE, M. K. DAS, A. ROY, AND C. P. J. GLAUDEMANS, *Mol. Immunol.*, 18 (1981) 277–280.
4 P. KOVÁČ AND C. P. J. GLAUDEMANS, *Carbohydr. Res.*, 123 (1983) c29–c30; *J. Carbohydr. Chem.*, 3 (1984) 349–358, and other papers in this series.
5 M. A. NAVIA, D. M. SEGAL, E. A. PADLAN, D. R. DAVIES, D. N. RAO, S. RUDIKOFF, AND M. POTTER, *Proc. Natl. Acad. Sci. U.S.A.*, 76 (1979) 4071–4074.
6 P. J. GAREGG AND T. NORBERG, *Acta Chem. Scand., Ser. B*, 33 (1979) 116–118, and papers cited therein.
7 P. KOVÁČ, C. P. J. GLAUDEMANS, W. GUO, AND T. C. WONG, *Carbohydr. Res.*, 140 (1985) 299–311; see also: P. KOVÁČ AND C. P. J. GLAUDEMANS, *Carbohydr. Res.*, 140 (1985) 277–288, and M. BERTOLINI AND C. P. J. GLAUDEMANS, *Carbohydr. Res.*, 15 (1970) 263–270.
8 M. E. JOLLEY AND C. P. J. GLAUDEMANS, *Carbohydr. Res.*, 33 (1974) 377–382.
9 O. OUCHTERLONY, *Acta Pathol. Microbiol. Scand.*, 25 (1948) 186–191.
10 N. ROY AND C. P. J. GLAUDEMANS, *Carbohydr. Res.*, 63 (1978) 318–322.
11 D. H. BUSS AND I. J. GOLDSTEIN, *J. Chem. Soc., C*, (1968) 1457–1461.
12 M. E. JOLLEY, S. RUDIKOFF, M. POTTER, AND C. P. J. GLAUDEMANS, *Biochemistry*, 12 (1973) 3039–3044.
13 M. POTTER AND C. P. J. GLAUDEMANS, *Methods Enzymol.*, 47 (1972) 388–394.
14 G. EKBORG, Y. SONE, AND C. P. J. GLAUDEMANS, *Carbohydr. Res.*, 110 (1982) 55–67.
15 P. KOVÁČ, E. A. SOKOLOSKI, AND C. P. J. GLAUDEMANS, *Carbohydr. Res.*, 128 (1984) 101–109.