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A synthetic heptasaccharide reveals the capability of a monoclonal antibody to read internal epitopes of a polysaccharide antigen

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ABSTRACT

The binding of the synthetic heptasaccharide, β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-(1→6)-β-D-Gal*p*-1-OCH_3 (**10**) with two monoclonal IgAs of the X24 gene-family has been investigated. The ligand **10** was synthesized by silver triflate mediated coupling of *O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl)-(1→3)-2,4,6-tri-*O*-benzoyl-α-D-galactopyranosyl chloride (**5**) to the benzoylated, *all*-β-(1→6)-linked methyl galactotetraoside **13**, having O-6⁴ free, followed by debenzoylation of the formed, fully protected methyl galactoheptaoside. The blockwise synthesis of the nucleophile **13** from readily available monosaccharides, and the synthesis of **5** from the corresponding β-1-*O*-benzoylated trisaccharide, is also described. Heptasaccharide **10** binds with the (1→6)-β-D-galactan-specific monoclonal antibodies X-24 and J539 with essentially the same K_a-values ($5.4 \times 10^5 M^{-1}$ and $6.4 \times 10^5 M^{-1}$, respectively) as does the methyl β-glycoside of *all*-β-(1→6)-linked galactotetraose **14** ($5.7 \times 10^5 M^{-1}$ and $5.9 \times 10^5 M^{-1}$, respectively). Of the series of homologous oligosaccharides studied previously (di- through a hexa-saccharide), **14** was found to show the highest affinity of interaction with both these immunoglobulins. The β-(1→3)-linked galactotriose, which forms the bulky terminus of **10**, does not appear to bind to these IgAs. Thus, the observation that the affinity of **10** is the same as that of **14** confirms that these immunoglobulins bind internal tetrasaccharide sequences of the antigenic (1→6)-β-D-galactopyranan.

INTRODUCTION

Of all monoclonal immunoglobulin-antigen binding studies, those done on the system IgA J539–(1→6)-β-D-galactopyranan are probably the most extensive. From the original work[1] on the antibody, to genetic studies[2] including site-specific mutagenesis[3], and thorough studies with specifically modified oligoasccharides that are forced to frame-shift along the antibody's subsites in order to bind (reviewed in refs. 4 and 5), detailed deductions on the binding mode could be made. Because of our long commitment to this work, we felt it essential to make certain of our deduction that the antibodies involved are capable of binding internal antigenic epitopes ("groove-binding" type[6]). We showed that this appeared to be so[7] with our finding that IgA J539 binds

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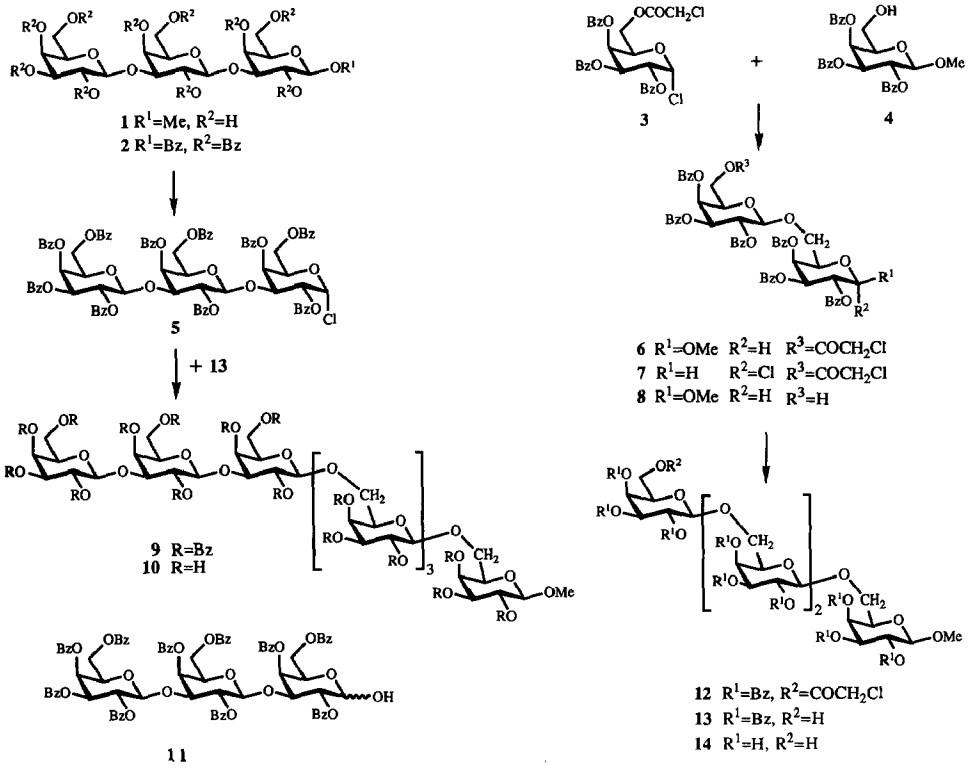
internal galactosyl sequences of a (1→6)-β-D-galactopyranan isolated from *P. zopfi*. In the unlikely event that a low incidence of branching was missed in the report[8] on the structure of the polysaccharide, our conclusion about its mode of binding to our antibody could be erroneous. Thus, we felt the necessity to establish the binding characteristics of these antibodies using well defined synthetic ligands. We previously showed[9] that methyl 3-*O*-β-D-galactopyranosyl-β-D-galactopyranoside does not bind to this class of immunoglobulins. We now report the construction of a heptasaccharide consisting of a β-(1→3)-linked-D-galactotriose terminus (by itself apparently non-binding) linked to the methyl β-glycoside of β-(1→6)-linked galactotetraose by a β-(1→6)-linkage. Binding studies on this heptasaccharide should reveal the capability of the IgA J539 class of monoclonal antibodies to bind to *internal* sequences of β-(1→6)-linked D-galactopyranosyl residues.

RESULTS AND DISCUSSION

Synthesis. — For the preparation of the target galactoheptaoside **10**, we chose a blockwise approach. This made it possible to reduce the number of steps compared to that necessary for the construction of the same structure in a stepwise manner. The synthesis required the fully benzoylated β-(1→6)-linked galactotriosyl chloride **5** as the glycosyl donor and the β-(1→3)-linked methyl galactotetraoside **13** as the glycosyl acceptor. Compound **5** was readily prepared (86%) by treatment of **2** (ref. 10) with dichloromethyl methyl ether (DCMME) in the presence of a catalytic amount of zinc chloride. Silver trifluoromethanesulfonate (silver triflate)-promoted coupling of 6-*O*-chloroacetyl-2,3,4,-tri-*O*-benzoyl-α-D-galactopyranosyl chloride (**3**) with methyl 2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside (**4**), both readily available from methyl β-D-galactopyranoside[11,12], gave the β-(1→6)-linked disaccharide **6** (95%). The latter was converted directly into the corresponding crystalline chloride **7** (81%) and the nucleophile **8** (94%) by treatment with the DCMME–$ZnCl_2$ reagent[13] and thiourea, respectively. Condensation of **7** and **8** gave the galactotetraoside **12** (89%), and deprotection of **12** at position O-6[4] yielded the nucleophile **13** (88%). The silver triflate-promoted condensation of the building blocks **5** and **13** was accompanied by extensive side reactions. Consequently, the pure, fully protected methyl galactoheptaoside **9** was isolated in 29% yield only, following two-stage chromatography with different solvent systems. In the first stage the desired product **9** was separated from a mixture of the starting nucleophile **13** and trisaccharide **11** (formed by hydrolysis of **5**). The n.m.r. (^{1}H- and ^{13}C-) spectra of **9** thus obtained showed, *inter alia*, signals indicating the presence of a small amount of an impurity. This contaminant was completely removed (n.m.r.) by chromatography using a different solvent system (see Experimental). Most of the unchanged **13** could be recovered when the aforementioned unresolved mixture of **13** and **11** was treated with chloroacetyl chloride and 2,4,6-trimethylpyridine, and the crude product was chromatographed. The 6[4]-*O*-chloroacetylated galactotetraoside **12** was then isolated in 59% yield, making the yield of **9** ~ 70% based on the amount of unrecovered nucleophile **13**. The weakly nucleophilic hemiacetal hydroxyl group in **11**, present in the mixture, did



not react under the conditions of chloroacetylation. The structure of the isolated **11** was supported by its n.m.r. spectra. Debenzoylation of **9** with a catalytic amount of sodium methoxide in toluene-methanol, followed by final purification of the deblocked compound by chromatography, gave heptasaccharide **10** in essentially theoretical yield.

^{13}*C-N.m.r. spectroscopy.* — Generally, carbon-signal assignments were made by mutual comparison of the spectra, and by comparison with spectra of related substances[14,15]. The carbon spectra of heptasaccharides **9** and **10** (Fig. 1) showed seven distinct resonances in the anomeric region. This, and other features of the spectra (see below), proved the presence of seven β-linked galactose units. Complete analysis of the spectrum of the fully benzoylated methyl heptaoside **9** was not possible, but from attached proton test (APT) experiments[16] it was evident, in accord with the structure **9**, that it contained three signals for methylene carbons bearing benzoyloxy groups (δ 62.75, 62.44, and 61.60), and four signals for methylene carbons carrying glycosyloxy substituents [δ 67.53 (2 C), 67.18, and 66.69]. Assignment of lines in the spectrum of **10** (Fig. 2) was made by comparison with the spectra of methyl tetraoside **14** (ref. 15) and methyl trioside **1** (ref. 14). Peaks in the spectra of **14** and **1** corresponding to carbon atoms of which the chemical environment was not expected to change significantly when they became parts of heptasaccharide **10** (all carbon atoms except C-1^{I} and C-2^{I} of compound **1** and C-5^{4} and C-6^{4} of **14**) were found in the spectrum of **10** at nearly identical δ values. Six of the seven anomeric carbon atoms in the spectrum of **10** could be

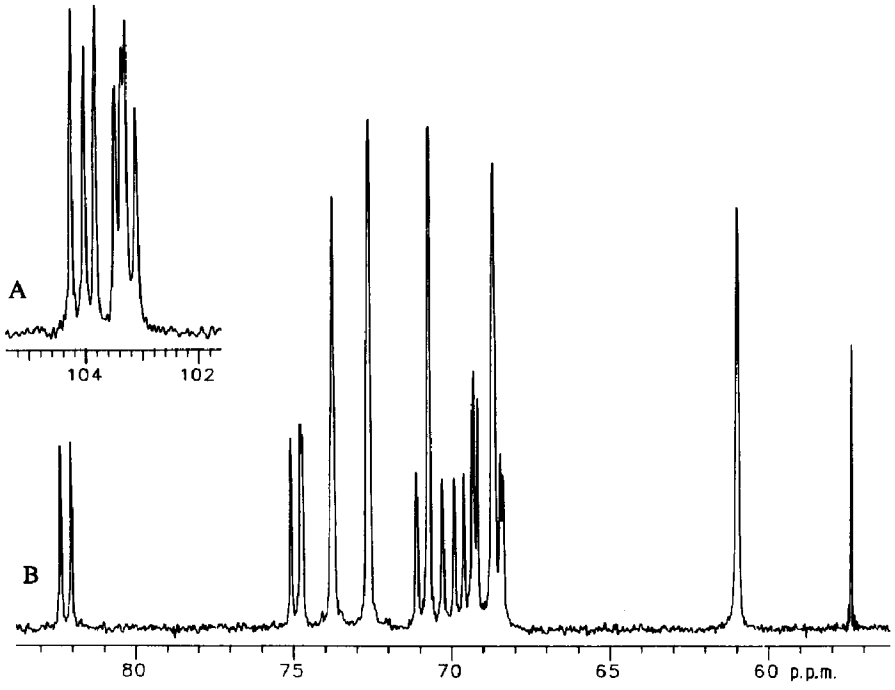


Fig. 1. ^{13}C-N.m.r. spectrum of compound **10**. A, Carbon atoms of the anomeric region, and B, of the non-anomeric region.

confidently assigned following the above reasoning. The most upfield of all anomeric signals (δ 103.1) in the spectrum of **10** was assigned to C-1[5], since the recorded upfield shift (from δ 103.57 in **1** to δ 103.1 in **10**) reflects, in terms of chemical shifts, the transition from a methoxyl group at C-1[1] in **1** to a glycon group in **10**, with the newly formed interglycosidic linkage being β-(1→6). Thus, for **10** (*cf.* the spectrum of **14**), $\delta_{C\text{-}1^1}$ at 103.86 p.p.m. − $\delta_{C\text{-}1^2}$ at 103.42 p.p.m. gives a $\Delta\delta$ of 0.44 p.p.m.; and the difference between the $\delta_{C\text{-}1^1}$ for **1** and the chemical shift of the most downfield anomeric signal in the spectrum of **10** is 0.46 p.p.m.

Binding studies. — Immunoglobulins capable of binding *only* to the non-reducing termini of saccharide antigenic epitopes have been called "cavity" binders, as opposed to so-called "groove" binders. The latter term refers to antibodies capable of binding to either internal or terminal segments[6]. Since our deductions about the binding patterns of monoclonal antibodies belonging to the X24 gene family[4,5,17] are based partly on their capability to bind to internal epitopes of a galactan antigen[7], it became important to unequivocally verify our prior deduction. To do so we construct a galactopyranosyl oligosaccharidic sequence wherein the nonreducing end consisted of a few residues posessing a linkage type that caused them, by themselves, to be unable to bind to the antibody, while the remainder consisted of the optimally binding β-(1→6)-linked tetramer. Thus the net antibody-affinity for the entire epitope would reveal the antibody-accessibility to that part of the epitope whose structure *did* reflect binding poten-

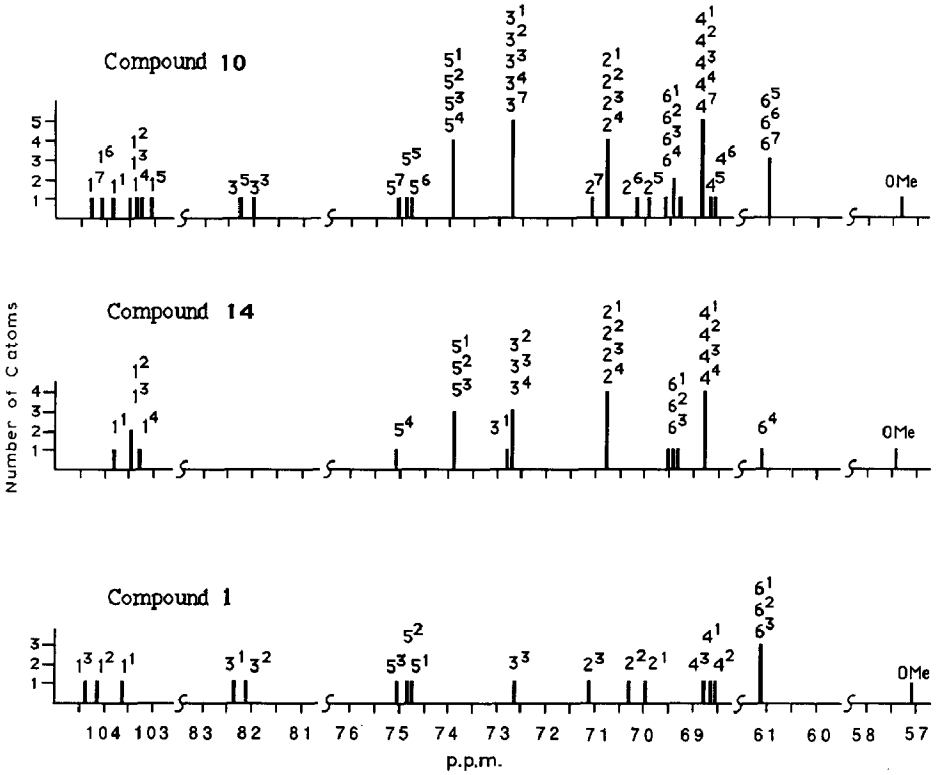


Fig. 2. Comparison of the ^{13}C-n.m.r. line spectra of compounds **1** (ref. 14), **14** (ref. 15), and **10**.

tial. In an extension of our past work[9] on the D-galactopyranosyl-(1→3)-β-D-galactopyranose sequence, we here showed that the trisaccharide **1** does not appear to bind to IgA J539 (Table I). Our observation that our galacto-heptasaccharide, having a nonreducing sequence of β-(1→3)-linkages attached to the methyl β-glycoside of (1→6)-β-D-galactopyranotetraose, binds to the two monoclonal antibodies of the X24 family here investigated with affinities identical to those for the parent methyl (1→6)-β-D-galactotetraoside proves that the bulky terminus does not interfere with binding, *i.e.*, that these antibodies are not cavity, but groove binders. They are capable of binding to *internal* sequences of four β-(1→6)-linked D-galactopyranosyl residues, unlike the known IgAs that bind only to the nonreducing *terminal* of a polysaccharide[6,18]. The possibility that IgA J539 binds only to the *reducing* end of a β-(1→6)-linked D-galactan can be ruled out from the observation that the β-(1→6)-linked D-galactan[8] from *P. zopfii* binds to the Fab' fragment of IgA J539 with a K_a equal to that of the maximally binding tetrasaccharide (0.59×10^6). In order to arrive at that K_a from the experimental data, the concentration of the polysaccharide had to be expressed as sequences of 30 galactosyl residues[7], *i.e.*: weight of polysaccharide per liter/mol. wt. of 30 anhydrogalactose residues. This means that the polysaccharide (with on the average 1200 galactosyl residues per chain[7]) is "read" by the antibody in terms of blocks of 30 sugar residues, and thus that each chain presents multiple binding sites to the antibody, *i.e.* the antibody

TABLE I

Binding constants of IgA J539 and IgA X24 with various ligands.

Compound[a]	$K_a(M^{-1})$	
	J539	*X24*
1	0[b]	0[b]
GalOMe[c]	1.0×10^3	0.5×10^3
Gal_2OMe[c]	4.7×10^4	2.1×10^4
Gal_3OMe[c]	4.8×10^5	3.2×10^5
Gal_4OMe[c](**14**)	5.9×10^5	5.7×10^5
10[d]	6.4×10^5	5.4×10^5

[a] GalOMe = methyl β-D-galactopyranoside, Gal_2OMe = methyl *O*-β-D-galactopyranosyl-(1→6)-β-D-galactopyranoside, *etc.* [b] No ligand-induced change in fluorescence, indicative of no binding. [c] K_a values are taken from ref. 5. [d] K_a values taken from Scatchard plots, Fig. 3.

can bind to internal sequences in the polysaccharide molecule. It can be readily appreciated that any polysaccharide has but one reducing end, no matter how many branch points may occur along its chain. Thus, if the antibody could bind to the tetrasaccharide at the reducing end of the polysaccharide *only* (and not to multiple segments of the polysaccharide molecule) the concentration used in the computation, in order to arrive at the known K_a, would have had to be that of the *entire* polysaccharide, and that was not the case[7].

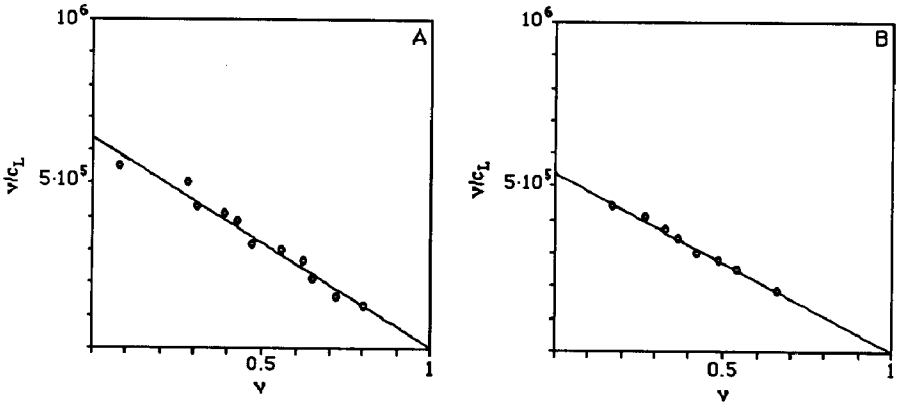


Fig. 3. Scatchard plots[20] for the binding of **10** to A, IgA J539, and B, IgA X24.

EXPERIMENTAL

General methods. — Optical rotations were measured at 25° with a Perkin–Elmer automatic polarimeter, Model 241 MC. Thin layer chromatography (t.l.c.) on precoated slides of Silica Gel G F254 (Analtech) was performed with solvent mixtures of appropriately adjusted polarity consisting of: *A*, carbon tetrachloride–acetone; *B*,

toluene–ethyl acetate; *C*, dichloromethane–acetone; *D*, 2-propanol–water–ethyl acetate; *E*, 1-propanol–water–ethyl acetate. Detection was effected by charring with 5% sulfuric acid in ethanol and, when applicable, with u.v. light. Preparative chromatography was performed on columns of Silica Gel 60 (Merck, No. 9385 or No. 15111). N.m.r. data were extracted from spectra measured at 25° with a Varian XL 300 spectrometer. Tetramethylsilane was used as internal standard for solutions in $CDCl_3$, methanol (δ_c 49.0) for those in D_2O. Proton signal assignments were made by first-order analysis of the spectra, and were supported by homonuclear decoupling experiments. Of the two magnetically nonequivalent geminal protons attached to a given primary carbon atom, the one resonating at lower field is denoted H-6a and the one resonating at a higher field H-6b. The superscripts used in reporting the n.m.r. data for oligosaccharides denote the sugar residues or groups containing the designated proton or carbon atom. These are serially numbered, beginning with the potential reducing residue. For example, H-1^2 refers to H-1 of the second sugar residue. Reactions requiring anhydrous conditions were performed under argon using common laboratory glassware, and reagents and solvents were handled with Hamilton Series 1000 gas-tight syringes. Unless stated otherwise, solutions in organic solvents were dried with anhydrous sodium sulfate, and concentrated at 2 kPa and 40°.

O-*(2,3,4,6-Tetra*-O-*benzoyl-β-*D-*galactopyranosyl)-(1→3)*-O-*(2,4,6-tri*-O-*benzoyl-β-*D-*galactopyranosyl)-(1→3)-2,4,6-tri*-O-*benzoyl-α-*D-*galactopyranosyl chloride* (**5**). — To a solution of **2** (ref. 10; 825 mg, 0.5 mmol) in a mixture of chloroform (5 mL) and dichloromethyl methyl ether (1 mL) was added freshly fused zinc chloride (~2 mg), and the mixture was stirred at 45–50°. After 2 h t.l.c. (solvent *A*, 3:1) showed complete conversion of **2** into a single, faster-moving product. After concentration, and coevaporation with toluene, the residue was chromatographed (solvent *A*, 10:1) to give **5** (675 mg, 86%) as a white foam, $[\alpha]_D$ +110° (*c* 0.7, $CHCl_3$); ^{1}H-n.m.r. ($CDCl_3$): δ 6.442 (d, 1 H, $J_{1,2}$ 3.9 Hz, H-1^1), 6.001 (br. d, 1 H, $J_{4,5}$ < 1 Hz, H-4^1), 5.987 (br. d, 1 H, $J_{3,4}$ 3.2, $J_{4,5}$ < 1 Hz, H-4^3), 5.823 (br. d, 1 H, $J_{4,5}$ < 1 Hz, H-4^2), 5.523 (dd, 1 H, $J_{2,3}$10 Hz, H-2^1), 5.473 (dd, 1 H, $J_{1,2}$ 7.7, $J_{2,3}$ 10.4 Hz, H-2^3), 5.379 (dd, 1 H, $J_{1,2}$ 7.9, $J_{2,3}$ 10 Hz, H-2^2), 5.278 (dd, 1 H, H-3^3), 4.970 (d, 1 H, H-1^2), 4.859 (d, 1H, H-1^3), 4.679 (dd, 1 H, $J_{5,6a}$ 6.2, $J_{6a,6b}$ 11.2 Hz, H-6^3a), 4.306 (dd, 1 H, $J_{5,6a}$ 4.9, $J_{6a,6b}$ 11.5 Hz, H-6^2a), 4.623–4.437 (m, 4 H, H-3^1, 3^2, 6^1a,6^1b), 4.414–4.356 (m, 2 H, H-6^2b,6^3b), and 4.247–4.138 (m, 3 H, H-5^1,5^2,5^3); ^{13}C-n.m.r. ($CDCl_3$): δ 101.59, 101.47 (C-1^2,1^3), 92.06 (C-1^1), 77.03 (C-3^2), 72.37 (C-3^1), 72.08 (C-2^2), 71.49, 71.43 (C-4^2,3^3), 71.18, 70.80, 70.52, 70.34 (C-4^1, 5^1, 5^2, 5^3), 70.04 (C-2^1), 69.52 (C-2^3), 67.61 (C-4^3), 62.79 (C-6^2), 62.57 (C-6^1), and 61.70 (C-6^3).

Anal. Calc. for $C_{88}H_{71}ClO_{25}$: C, 67.58; H, 4.58; Cl, 2.27. Found: C, 66.82; H, 4.61; Cl, 2.21.

Methyl O-*(2,3,4-tri*-O-*benzoyl-6*-O-*chloroacetyl-β-*D-*galactopyranosyl)-(1→6)-2,3,4-tri*-O-*benzoyl-β-*D-*galactopyranoside* (**6**). — A solution of 2,3,4-tri-*O*-benzoyl-6-*O*-chloroacetyl-α-D-galactopyranosyl chloride[11] (**4**; 1.46 g, 2.5 mmol), methyl 2,3,4-tri-*O*-benzoyl-β-D-galactopyranoside[11] (**5**; 1.01 g, 2.0 mmol) and 2,4,6-trimethylpyridine (267 mg, 2.2 mmol) in dichloromethane (10 mL) was added dropwise at room temperature to a stirred suspension of silver triflate (0.72 g, 2.8 mmol) in dichloromethane (5

mL). After 10 min the solution turned acidic, and after further 5 min it was neutralized with 2,4,6-trimethylpyridine. T.l.c. (solvent *A*, 10:1) showed complete conversion of the starting material into one major product. The mixture was filtered, the solids were washed with dichloromethane, and the combined filtrates were washed with aqueous sodium thiosulfate and concentrated. The crystalline residue was twice recrystallized from dichloromethane–ethanol to give **6** (2.00 g, 95%), m.p. 254–255°, $[\alpha]_D$ +166° (*c* 1.4, $CHCl_3$); ^{1}H-n.m.r. ($CDCl_3$):δ 5.89 (br. d, 1 H, $J_{3,4}$ 3.3, $J_{4,5}$ > 1 Hz, H-4^1) 5.84 (br. d, 1 H, $J_{3,4}$ 3.3, $J_{4,5}$ < 1 Hz, H-4^2), 5.77, (dd, 1 H, $J_{1,2}$ 7.8, $J_{2,3}$ 10.5 Hz, H-2^2), 5.70 (dd, 1 H, $J_{1,2}$ 7.8, $J_{2,3}$ 10.5 Hz, H-2^2), 5.54, (dd, 1 H, H-3^2), 5.52 (dd, 1 H, H-3^1), 4.87 (d, 1 H, H-1^2), 4.57 (d, 1 H, H-1^1), 4.24–4.12 (m, 6 H, H-5^1,5^2, 6^{1}a, 6^{1}b, 6^{2}a, 6^{2}b), 3.94 (s, 2 H, C*H*$_2$Cl), and 3.26 (s, 3 H, OC*H*$_3$); ^{13}C-n.m.r. ($CDCl_3$): δ 102.2 (C-1^1), 101.4 (C-1^2), 73.2, 71.0 (C-5^1,5^2), 71.7 (C-3^1), 71.5 (C-3^2), 69.8 (C-2^1), 69.6 (C-2^2), 68.7 (C-4^1); 68.2 (C-6^1), 67.8 (C-4^2), 63.1 (C-6^2), 56.8 (O*C*H$_3$), and 40.4 (*C*H$_2$Cl).

Anal. Calc. for $C_{57}H_{49}ClO_{19}$: C, 64.74; H, 4.67; Cl, 3.35. Found: C, 64.67; H, 4.69; Cl, 3.42.

O-*(2,3,4-Tri*-O-*benzoyl-6*-O-*chloroacetyl-β*-D-*galactopyranosyl)-(1→6)-2,3,4-tri*-O-*benzoyl-α*-D-*galactopyranosyl chloride* (**7**). — To a solution of **6** (2.12 g, 2.0 mmol) in a mixture of chloroform (20 mL) and dichloromethyl methyl ether (8 mL) was added freshly fused zinc chloride (~30 mg), and the mixture was stirred for 4.5 h at 60°, when t.l.c. (solvent *B*, 10:1) showed nearly complete conversion of the starting material into a major, faster moving product. After workup, as described for the preparation of **5**, and chromatography (solvent *A*, 15:1), the crystalline material was recrystallyzed twice from acetone–diethyl ether to give **7** (1.72 g, 81%), m.p. 229°, $[\alpha]_D$ +173.5° (*c* 1.1, $CHCl_3$; ^{1}H-n.m.r. ($CDCl_3$): δ 6.44 (d, 1 H, $J_{1,2}$ 3.9 Hz, H-1^1), 6.02 (br. d, 1 H, $J_{3,4}$ 3.4, $J_{4,5}$ < 1 Hz, H-4^1), 5.96 (dd, 1 H, $J_{2,3}$ 10.5 Hz, H-3^1), 5.82 (br. d, 1 H, $J_{3,4}$ 3.4, $J_{4,5}$ < 1 Hz, H-4^2), 5.76–5.71 (m, 2 H, H-2^1,2^2), 5.52 (dd, 1 H, $J_{2,3}$ 10.4 Hz, H-3^2), 4.87 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1^2), 4.74 (br. t, 1 H, H-5^1), 4.15 (s, 1 H, C*H*$_2$Cl), 4.12–4.08, and 3.95–3.90 (2 m, 5 H, H-5^2,6^{1}a,6^{1}b,6^{2}a,6^{2}b); ^{13}C-n.m.r. ($CDCl_3$): δ 101.45 (C-1^2), 91.35 (C-1^1), 71.71, 71.57 (C-5^1,3^2), 71.10 (C-5^2), 69.47 (C-2^2), 68.93, 68.50 (C-3^1,4^1), 67.97, 67.80 (C-2^1,4^2), 67.23 (C-6^1), 63.09 (C-6^2), and 40.47 (*C*H$_2$Cl).

Anal. Calc. for $C_{56}H_{46}Cl_2O_{17}$: C, 63.34; H, 4.37; Cl, 6.68. Found: C, 63.21; H, 4.34; Cl, 6.60.

Methyl O-*(2,3,4-tri*-O-*benzoyl-β*-D-*galactopyranosyl)-(1→6)-2,3,4-tri*-O-*benzoyl-β*-D-*galactopyranoside* (**8**). — To a suspension of **6** (1.06 g, 1.0 mmol) in chloroform–methanol 1:1 (40 mL) was added, at 50°, thiourea (0.30 g, 4.0 mmol). After 4 h at 50° a clear solution was formed, and after a total of 10 h t.l.c. (solvent *B*, 10:1) showed complete conversion of **6**. The mixture was cooled to room temperature, the solution was diluted with dichloromethane, washed successively with M HCl, 5% aqueous sodium hydrogencarbonate, and water, dried, and concentrated. The residue was crystallized from dichloromethane–methanol to give **8** (0.93 g, 94%), m.p. 139°, $[\alpha]_D$ +184° (*c* 1.3, $CHCl_3$); lit.[10] m.p. 139°, $[\alpha]_D$ +186°.

Methyl O-*(2,3,4-tri*-O-*benzoyl-6*-O-*chloroacetyl-β*-D-*galactopyranosyl)-(1→6)*-O-*(2,3,4-tri*-O-*benzoyl-β*-D-*galactopyranosyl)-(1→6)*-O-*(2,3,4-tri*-O-*benzoyl-β*-D-*ga-*

*lactopyranosyl)-(1→6)-2,3,4-tri-*O*-benzoyl-β-*D*-galactopyranoside* (**12**). — A mixture of **7** (1.31 g, 1.23 mmol), **8** (1.08 g, 1.10 mmol), 2,4,6-trimethylpyridine (133 mg, 1.10 mmol), and silver triflate (385 mg, 1.50 mmol) in dichloromethane (17 mL) was treated as described for the preparation of **6**. After processing as described for **6**, and chromatography (solvent *A*, 20:1), **12** (1.96 g, 89%) was obtained as an amorphous solid, $[\alpha]_D$ +109° (*c* 0.6, $CHCl_3$); ^{1}H-n.m.r. ($CDCl_3$), definite signals: δ 5.925, 5.875, 5.835, 5.765 (4 br. d, 4 × 1 H, $J_{3,4}$ 3 Hz, H-4^1,4^2,4^3,4^4), 4.79 (d, 1 H, $J_{1,2}$ 7.9 Hz, H-1^4), 4.575, 4.552, 4.500 (3 d, 3 × 1 H, $J_{1,2}$ 7.8 Hz, H-1^1,1^2,1^3), 3.965 (s, 2 H, CH_2Cl), and 3.234 (s, 3 H, OCH_3); ^{13}C-n.m.r. ($CDCl_3$): δ 102.18 (C-1^1), 101.22, 100.89 (C-1^2,1^3), 100.76 (C-1^4), 72.98 (C-5^1), 72.36 (2 C, C-5^2,5^3), 71.75, 71.55 (3 C, 1 C, C-3^1,3^2,3^3,3^4), 71.01 (C-5^4), 69.92 (3 C, C-2^2,2^3,2^4), 69.71 (C-2^1), 68.66 (C-4^1),67.93 (C-6^1), 67.80 (3 C, C-4^2,4^3,4^4), 66.72, 66.15 (C-6^2,6^3), 63.04 (C-6^4), 56.77 (OCH_3), and 40.45 (CH_2Cl).

Anal. Calc. for $C_{111}H_{93}ClO_{34}$: C, 66.45; H, 4.72; Cl, 1.77. Found: C, 66.29; H, 4.76; Cl, 1.95.

Methyl O-*(2,3,4-tri-O-benzoyl-β-*D*-galactopyranosyl)-(1→6)-*O*-(2,3,4-tri-*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* (**13**). — To a solution of **12** (1.27 g, 0.63 mmol) in chloroform (20 mL) was added thiourea (0.19 g, 2.5 mmol) in methanol (10 mL), and the solution was kept at 50°. After 25 h t.l.c. (solvent *A*, 3:1) showed complete conversion of **12**. After usual processing, chromatography (solvent *A*, 5:1) gave **13** (1.07 g, 88%) as an amorphous solid, $[\alpha]_D$ +113° (*c* 1.1, $CHCl_3$), lit.[15] $[\alpha]_D$ +121°; ^{1}H- and ^{13}C-n.m.r. spectra were consistent with those reported[15].

Methyl O-*(2,3,4,6-tetra-*O*-benzoyl-β-*D*-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-benzoyl-β-*D*-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-benzoyl-β-*D*-galactopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-benzoyl-β-*D*-galactopyranosyl)-(1→6)-*O*-(2,3,4-tri-*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* (**9**). — A solution of **13** (733 mg, 0.38 mmol), **5** (676 mg, 0.43 mmol), and 2,4,6-trimethylpyridine (48.5 mg, 0.40 mmol) in dichloromethane (5 mL) was added at room temperature to a stirred suspension of silver triflate (128 mg, 0.50 mmol) in dichloromethane (2 mL). Silver chloride precipitated immediately, and the solution turned acidic to litmus after 30 min. T.l.c. (solvent *C*, 20:1) indicated complete conversion of **5**. After neutralization with 2,4,6-trimethylpyridine, and workup as described for the preparation of **6**, the residue was chromatographed (solvent *C*, gradient 40:1 to 20:1) to give first crude **9** (437 mg).

This was followed by a mixture of **13** and **11** (641 mg), the latter having been formed by hydrolysis of **5**, which was verified as follows. The mixture was dissolved in dichloromethane (20 mL) and treated with 2,4,6-trimethylpyridine (121 mg, 1.0 mmol) and chloroacetyl chloride (113 mg, 1.0 mmol) for 2 h at r.t. T.l.c. (solvent *A*, 3:1) showed the presence of a faster moving product having the same mobility as **12**, and a slower moving one, which was detected with phthalic acid–aniline reagent[19], indicative of a reducing sugar. The reaction mixture was partitioned between dichloromethane and 5% aqueous $NaHCO_3$ solution, and the organic phase was dried (Na_2SO_4) and concentrated. Chromatography (solvent *A*, 10:1), gave first **12** (452 mg, 59%), $[\alpha]_D$ +101.4° (*c* 0.5, $CHCl_3$); ^{1}H-n.m.r. ($CDCl_3$) data were consistent with those reported above.

Eluted next was *O*-(2,3,4,6-tetra-*O*-benzoyl-β-D-galactopyranosyl)-(1→3)-*O*-(2,4,6-tri-*O*-benzoyl-β-D-galactopyranosyl)-(1→3)-2,4,6-tri-*O*-benzoyl-D-galactopyranose (**11**; 33 mg, 5%; *a*:β ~5:1), ^{1}H-n.m.r. ($CDCl_3$): δ 5.99, 5.96 (2 br. d, 2 H, $J_{3,4}$ 3.5, $J_{3,4}$ 3.2 Hz, H-4^1*a*,4^{3}β), 5.91 (br. d, 1 H, $J_{3,4}$ 3.7 Hz, H-4^{1}β), 5.82 (br. d, 1 H, $J_{3,4}$ 2.8 Hz, H-4^2*a*,4^{2}β), 5.60 (d, 1 H, $J_{1,2}$ 3.6 Hz, H-1^1*a*), 5.49–5.33 (m, 3 H, H-2^1*a*,2^2*a*,2^3*a*,2^{1}β,2^{2}β,2^{3}β), 5.26 (dd, 1 H, $J_{2,3}$ 10.5 Hz, H-3^3*a*,3^{3}β), 4.99 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1^2*a*), 4.62 (d, 1 H, $J_{1,2}$ 7.8 Hz, H-1^{2}β), 4.85 (br. d, 1 H, $J_{1,2}$ 7.7 Hz, H-1^3*a*, 1^{3}β), 4.80 (br. d, 1 H, $J_{1,2}$ 7.5 Hz, H-1^{1}β), 4.70–4.25 (m, 7 H, H-6^{1}a,6^{1}b,6^{2}a,6^{2}b,3^1,3^2,5^3, both anomers), and 4.19–4.10 (m, 4 H, H-5^1,5^2,6^{3}a,6^{3}b, both anomers); ^{13}C-n.m.r. ($CDCl_3$), definite signals: δ 101.66 (C-1^2*a*,1^{2}β), 101.41 (C-1^3*a*,1^{3}β), 96.20 (C-1^{1}β), and 90.57 (C-1^1*a*).

Compound **9**, obtained as described above, appeared homogeneous by t.l.c. in solvent *C* (20:1) but contained two minor, faster moving impurities, as shown by t.l.c. using solvent *A* (2:1). Rechromatography (solvent *A*, gradient 15:1 to 5:1) gave pure **9** (377 mg, 29%, or ~70% based on the amount of **13** consumed) as a white foam, $[a]_D$ +89.5° (*c* 0.5, $CHCl_3$); ^{13}C-n.m.r. ($CDCl_3$): δ 102.05 (C-1^1), 101.70, 101.38, 101.22, 101.07, 100.98, 100.82 (C-1^2,1^3,1^4,1^5,1^6,1^7), 76.71, 76.63 (C-3^5,3^6), 72.68 (2 C, C-5^2,5^5), 72.33 (C-5^1), 72.02, 71.87, 71.73, 71.68, 71.49 (2 C, 3 C, 1 C, 1 C, 2 C, C-2^5,2^6,3^1,3^2,3^3,3^4,5^3,5^4,5^6), 71.21, 71.11 (C-4^5,5^7), 70.11, 70.03 (1 C, 4 C, C-2^2,2^3,2^4,3^7,4^6), 69.95 (C-2^1), 69.48 (C-2^7), 68.63 (C-4^1), 67.94 (3 C, C-4^2,4^3,4^4), 67.53 (3 C, C-4^7,6^1,6^4), 67.18 (C-6^3), 66.69 (C-6^2), 62.75 (C-6^6), 62.44 (C-6^5), 61.60 (C-6^7), and 56.65 (O*C*H$_3$).

Anal. Calc. for $C_{197}H_{162}O_{58}$: C, 68,44; H, 4.72. Found: C, 68.17; H, 4.83.

Methyl O-β-D-*galactopyranosyl-(1→3)*-O-β-D-*galactopyranosyl-(1→3)*-O-β-D-*galactopyranosyl-(1→6)*-O-β-D-*galactopyranosyl-(1→6)*-O-β-D-*galactopyranosyl-(1→6)*-O-β-D-*galactopyranosyl-(1→6)*-β-D-*galactopyranoside* (**10**). — To a warm solution of **9** (346 mg, 0.1 mmol) in 1:5 toluene–methanol (30 mL) was added sodium methoxide in methanol (M, 0.5 mL), and the clear solution was kept at 50°, with occasional swirling, for 16 h. After cooling the mixture to room temperature, the accumulated precipitate was centrifuged off and washed twice by suspending it in methanol and centrifuging, to give crude **10** and alkaline supernatants. Chromatography of the collected **10** (solvent *E*, gradient 3:1.5:0.5 to 3:2.5:0.5) gave material that was treated with water (~7 mL), then some undissolved material was centrifuged off, and the solution of **10** was lyophilized. The residue was redissolved in water (~1 mL), filtered through a membrane filter (Millipore Millex GV4, 0.22 μm, Cat. No. SLGVL04OS), and lyophilized again to give **10** (95 mg). The combined alkaline supernatants were neutralized with Dowex 50 (H^+)resin, concentrated, and the residue was processed as described above to give additional **10** (19 mg). Both fractions were homogeneous by t.l.c. in solvent *D* (3:2:0.5); total yield 114 mg (98%), $[a]_D$ +4.5°, $[a]_{365}$ +15.1° (*c* 0.3, H_2O). Since **10** is a hygroscopic solid these values for optical rotation might be somewhat low. ^{13}C-N.m.r. spectroscopy of **10** in D_2O (Fig. 1) failed to reveal any impurity: δ 104.26 (C-1^7), 104.02 (C-1^6), 103.84 (C-1^1), 103.47 (C-1^5), 103.36, 103.30, (C-1^2,1^3), 103.11 (C-1^4), 82.38 (C-3^5), 82.04 (C-3^6), 75.09 (C-5^7), 74.79 (C-5^5), 74.73 (C-5^6), 73.78 (4 C, C-5^1,5^2,5^3,5^4), 72.63 (5 C, C-3^1,3^2,3^3,3^4,3^7), 71.12 (C-2^7), 70.74 (4 C, C-2^1,2^2,2^3,2^4), 70.30, 69.92 (C-2^5,2^6), 69.62, 69.33, 69.19 (1 C, 2 C, 1 C, C-6^1,6^2,6^3,6^4),

68.70 (5 C, C-4^1,4^2,4^3,4^4,4^7), 68.47 (C-4^5), 68.38 (C-4^6), 61.00 (3 C, C-6^5,6^6,6^7), and 57.39 (OCH_3).

Fluorescence titration. — Immunoglobulins J539 and X24 were used in aqueous phosphate-buffered saline (0.01M phosphate, 0.15M NaCl) at pH 7.4. Protein solutions were used at concentrations of 2.4×10^{-7}M and 3.7×10^{-7}M for IgA J539 and X24, respectively. The ligand stock solutions had concentrations of 1.3×10^{-4}M for compound **10** and 0.10M for compound **1**, respectively. Titration procedures and the generation of Scatchard plots were as reported previously[20] (Fig. 3).

REFERENCES

1 M. E. Jolley, S. Rudikoff, M. Potter, and C. P. J. Glaudemans, *Biochemistry*, 12 (1973) 3039–3044.
2 S. Rudikoff, D. N. Rao, C. P. J. Glaudemans, and M. Potter, *Proc. Natl. Acad. Sci. U.S.A.*, 77 (1980) 4270–4274.
3 S. R. Arepalli, S. Rudikoff, and C. P. J. Glaudemans, unpublished data.
4 C. P. J. Glaudemans and P. Kováč, *ACS Symp. Ser.*, 374 (1988) 78–108.
5 C. P. J. Glaudemans, *Mol. Immunol.*, 24 (1988) 371–377, and references therein.
6 J. Cisar, E. A. Kabat, M. M. Dorner, and J. Liao, *J. Exp. Med.*, 142 (1975) 435–459.
7 C. P. J. Glaudemans, A. K. Bhattacharjee, and B. N. Manjula, *Mol. Immunol.*, 23 (1986) 655–660.
8 D. J. Manners, I. R. Pennie, and J. F. Ryley, *Carbohydr. Res.*, 29 (1973) 63–67; W. Jack and R. J. Sturgeon, *ibid.*, 49 (1976) 335–340.
9 P. Kováč, C. P. J. Glaudemans, and R. B. Taylor, *Carbohydr. Res.*, 142 (1985) 158–164.
10 T. Ziegler, B. Adams, P. Kováč, and C. P. J. Glaudemans, *J. Carbohydr. Chem.*, in press.
11 T. Ziegler, P. Kováč, and C. P. J. Glaudemans, *Carbohydr. Res.*, 194 (1989) 185–198.
12 E. M. Nashed and C. P. J. Glaudemans, *J. Org. Chem.*, 52 (1987) 5255–5260.
13 H. Gross, I. Farkas, and R. Bognar, *Z. Chem.*, 18 (1978) 201–210.
14 P. Kováč, R. B. Taylor, and C. P. J. Glaudemans, *J. Org. Chem.*, 50 (1985) 5323–5333.
15 P. Kováč, *Carbohydr. Res.*, 153 (1986) 237–251.
16 S. L. Patt and J. N. Shoolery, *J. Magn. Reson.*, 46 (1982) 535–539.
17 R. Ollo, C. Auffray, J. L. Sikorav, and F. Rougeon, *Nucleic Acids Res.*, 9 (1981) 4099–4109; A. B. Hartman and S. Rudikoff, *EMBO J.*, 3 (1984) 3023–3030.
18 C. P. J. Glaudemans, P. Kováč, and A. S. Rao, Carbohydr. Res., 190 (1989) 267–277.
19 *Anfärbereagenzien für Dünnschicht- und Papier-Chromatographie*, E. Merck AG, Darmstadt (F.R.G.), p. 3.
20 M. E. Jolley and C. P. J. Glaudemans, *Carbohydr. Res.*, 33 (1974) 377–382.