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Spacer-modified saccharides for the regioselective photoaffinity labelling of the binding site of an immunoglobulin

Jochen Lehmann*, and Markus Scheuring
Institut für Organische Chemie und Biochemie der Universität Freiburg i. Br., Albertstr. 21, D-7800 Freiburg i. Br. (Germany)

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ABSTRACT

The spacer-modified trisaccharides that mimic (1→6)-linked β-D-galactotetraose (Gal_4), namely, *O*-β-D-galactopyranosyl-(1→6)-*S*-β-D-galactopyranosyl-(1→11)-8-azi-6,7,8,9,10-pentadeoxy-11-thio-D-*galacto*-undecose (**12**) and *O*-β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→13)-8-azi-6,7,8,9,10,11,12-heptadeoxy-D-*galacto*-tridecose (**20**) were synthesised by coupling disaccharide derivatives with 8-azi-6,7,8,9,10-pentadeoxy-1,2:3,4-di-*O*-isopropylidene-11-*O*-tosyl-α-D-*galacto*-undecopyranose (**10**) and 8-azi-6,7,8,9,10,11,12-heptadeoxy-1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-tridecopyranose (**17**), respectively. Compounds **12** and **20** had affinities for the combining sites of the antibodies IgA X 24 and IgA J 539 similar to those of *O*-β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→11)-8-azi-6,7,8,9,10-pentadeoxy-D-*galacto*-undecose (**7**) and the native ligand Gal_4. Tritium-labelled **7** chemically modified the heavy and light chains of IgA J 539, whereas 8-azi-6,7,8,9,10-pentadeoxy-D-(11-^{3}H)*galacto*-undecose (**5a**) reacted only with the heavy chain.

INTRODUCTION

Many carbohydrate-binding proteins, such as endoglycanases, lectins, transport proteins for oligosaccharides, and antibodies directed against polysaccharides or glycoconjugates, recognise more or less extended oligosaccharide structures. The antigalactan antibody IgA J 539 binds (1→6)-linked β-D-galactopyranosyl units. The binding site can accommodate four Gal moieties in a specific order (Fig. 1) and with binding energies A > > B > C > D. Binding site A is the most effective[1] and is assumed to be occupied preferentially if only one or two Gal units are offered. From X-ray studies, it seems probable that the heavy and light chains meet near the binding sites B and A. This is also the area where two tryptophanyl residues are located, the perturbation of which by ligand binding is used as the basis for fluorescence measurements whereby binding constants can be determined accurately by titration with a potential ligand.

We have described syntheses and applications of two photoaffinity ligands, involving one[2] [3-azi-1-methoxybutyl β-D-galactopyranoside (**1**)] and two[3] [3,7-anhydro-2-azi-1,2-dideoxy-8-*O*-β-D-galactopyranosyl-D-*glycero*-L-*manno*-octitol (**2**)] Gal*p* units. The photolabile group of each of these compounds was part of the aglycon and the use of radioactive ligands revealed chemical modification of the heavy

* Author for correspondence.

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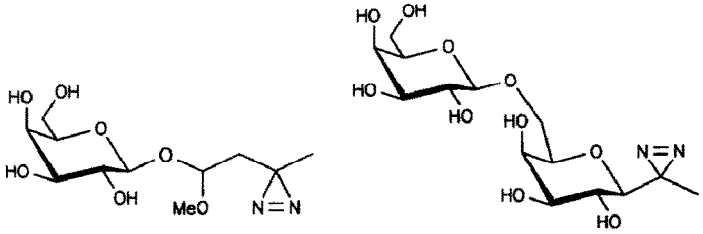

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and light chains of the antibody. This fact was explained by different modes of binding between receptor and ligands that involved different subsites and the flexibility of the aglycon. We have described also the synthesis[4] of a photolabile spacer-modified trisaccharide, namely, *O*-β-D-galactopyranosyl-(1→6)-*O*-β-D-galactopyranosyl-(1→11)-8-azi-6,7,8,9,10-pentadeoxy-D-*galacto*-undecose (**7**), which mimics the optimal ligand, the (1→6)-linked β-D-galactotetraose (Gal_4) and its methyl β-glycoside (Gal_4-OMe). Although **7** has only three Gal moieties, it binds as well as Gal_4. It is assumed that **7** will occupy preferentially the subsites A, C, and D (Fig. 1), so that subsite B will be modified chemically by reaction with the spacer.

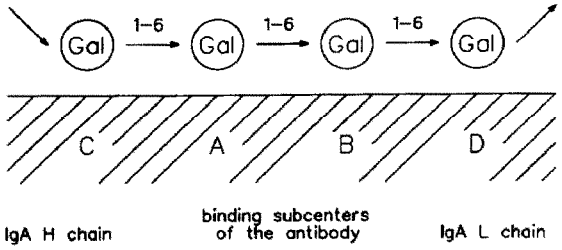


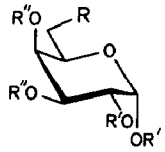
Fig. 1. Schematic drawing of the mode of binding of a (1→6)-linked β-D-galactopyranan segment to the antibody IgA J 539.

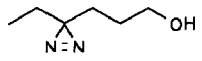
We now describe syntheses of radioactively labelled **7a*** and its "aglycon" moiety, the undecose **5a**, a spacer-modified monosaccharide, and their application for regioselective labelling of binding sites. The syntheses are also described of the thio analogue **12**, a new spacer-modified trisaccharide **20** with a 6-membered spacer, and its "aglycon", a tridecose **18**, in order to assess the effects of exchange of the heteroatom and the length of the spacer on binding.

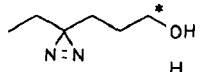
RESULTS

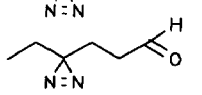
Syntheses of the spacer-modified monosaccharides **5a** *and* **18**. — Oxidation of 8-azi-6,7,8,9,10-pentadeoxy-1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-undecose (**3**)

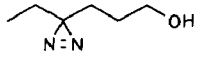
* Radiolabelled compounds are designated by **a**.

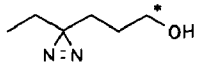


3 R′,R′ = R″,R″ = Ip, R = 

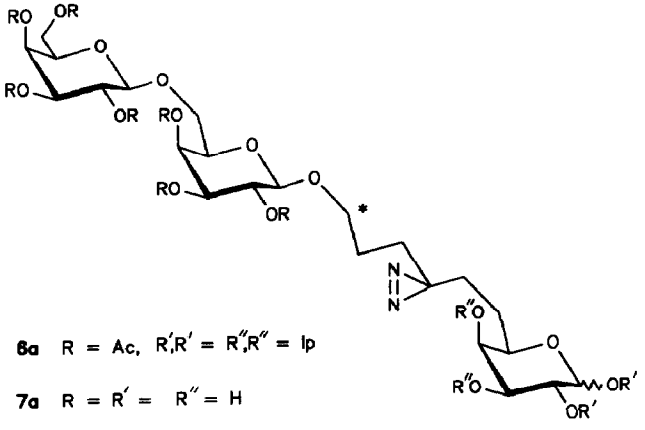
3a R′,R′ = R″,R″ = Ip, R = 

4 R′,R′ = R″,R″ = Ip, R = 

5 R′ = R″ = H, R = 

5a R′ = R″ = H, R = 

* ^{3}H-labelling



6a R = Ac, R′,R′ = R″,R″ = Ip

7a R = R′ = R″ = H

* ^{3}H-labelling

with pyridinium chlorochromate gave the aldehyde **4**, which was reduced with sodium borotritide to yield **3a**. Removal of the isopropylidene group from **3a** yielded **5a** as a potential ligand for affinity labelling. The tritium-labelled compound **6a**, which yielded **7a** after deblocking, had been prepared[4] from **3a**.

Compound **18** was prepared by a modification of the procedure published[5] for **5**. Chain elongation was carried out by reacting 6,7-dideoxy-1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-octodialdo-1,5-pyranose[5] **13** with the Grignard reagent obtained from 1-benzyloxy-5-bromopentane (prepared by reaction of the tosylate with lithium bromide) to yield 13-*O*-benzyl-6,7,9,10,11,12-hexadeoxy-1,2:3,4-di-*O*-isopropylidene-DL-*glycero*-α-D-*galacto*-tridecopyranose (**14**). Oxidation of the alcohol **14** with pyridinium

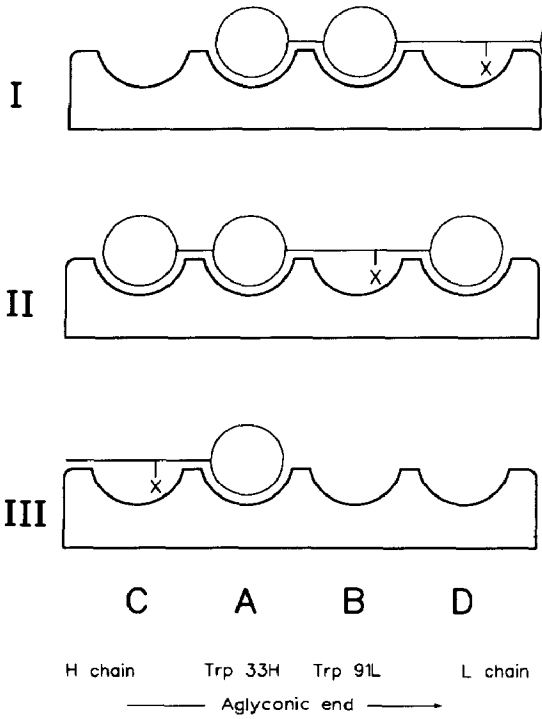


Fig. 2. Binding modes I–III (*cf.* Fig. 1).

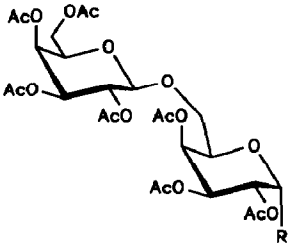
chlorochromate gave the ketone **15**. The diazirino group was introduced into **15** by the method described[5] to give **16**. Oxidative debenzylation of **16** with bromine[6] gave **17** which was further deblocked by mild acid hydrolysis to give **18**.

Syntheses of spacer-modified trisaccharides **12** *and* **20**. — The spacer was introduced as a replacement for a monosaccharide unit. For Gal_4, this replacement could be for either C-1, O-5, C-5, and C-6 by a 4-membered spacer as in **7**, or C-1/6 by a 6-membered spacer as in **20**. Each spacer-modified trisaccharide ought to bind to the four-subsite receptor (Fig. 2 II).

Glycosylation of **17** with the disaccharide glycosyl bromide **8** gave *O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-β-D-galactopyranosyl)-(1→13)-8-azi-6,7,8,9,10,11,12-heptadeoxy-1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-tridecopyranose (**19**) from which **20** was obtained.

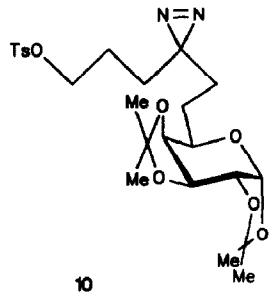
Glycosylation is sometimes effected more conveniently using a thiol[7]. Thus, the *S*-benzoyl derivative **9**, prepared from **8**, reacted with the tosylate (**10**) of **12** to give **11**, which, after deblocking, yielded the thio analogue (**12**) of **7**.

Binding studies and photoaffinity labelling. — The photolabile compounds **1**[2] and **2**[3] have about the same affinity for the binding site of IgA X 24 as methyl β-D-galactopyranoside and Gal_2, respectively (Table I). Compound **7**, which binds almost as well as Gal_4, provides another example of the "clustering effect", when structural elements, each with a low affinity, have mutually amplified binding capacities when



8 R = Br

9 R = SBz



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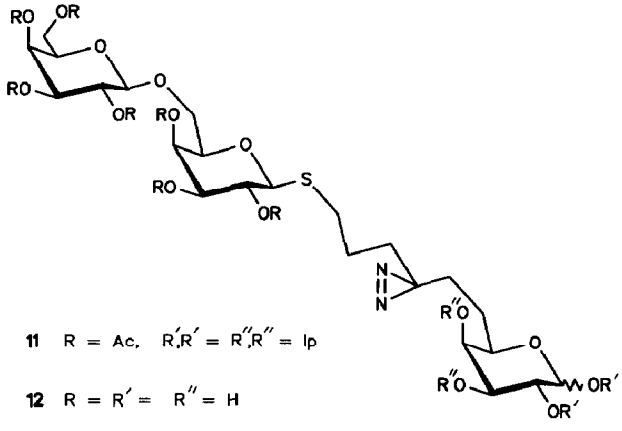


11 R = Ac, R′,R′ = R″,R‴ = Ip

12 R = R′ = R″ = H

TABLE I

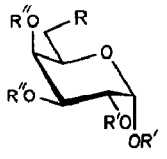
Binding constants of some ligands to antigalactan antibodies IgA J 539 and IgA X 24[1-4] measured by fluorescence titration[9]

Compound	K_i [M] *IgA J 539*	K_i [M] *IgA X 24*
Gal-OMe	1.0×10^{-3}	2.0×10^{-3}
Gal_2-OMe[a]	2.1×10^{-5}	4.7×10^{-5}
Gal_4-OMe[a]	1.7×10^{-6}	1.7×10^{-6}
Gal_2[b]	–	1.7×10^{-4}
Gal_4[b]	2.9×10^{-6}	3.7×10^{-6}
1	6.5×10^{-4}	5.7×10^{-4}
2	–	1.0×10^{-4}
5	6.3×10^{-3}[c]	6.9×10^{-3}[c]
7	3.1×10^{-6}	9.0×10^{-6}
12	–	2.0×10^{-5}
20	4.2×10^{-6}	9.7×10^{-6}

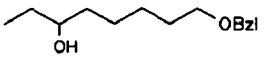
[a] Methyl glycoside of β-(1→6)-linked di- and oligo-saccharides. [b] β-(1→6)-Linked[10]. [c] May not be the true binding constants (see Discussion).

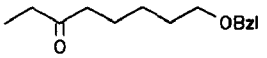
covalently linked[8]. Binding affinities can be measured accurately and easily by fluorimetry[9]. Perturbation of the tryptophanyl residues is caused apparently by all β-D-galactopyranosides and their homologues. Since a β-D-Gal*p* residue naturally occupies the subsite A, the binding studies give more or less the results expected. The spacer-modified monosaccharides behave like methyl β-D-galactopyranoside (Table I), and the two spacer-modified trisaccharides **12** and **20** have affinities similar to that[4] of **7**.

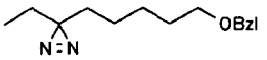
When the antibody was incubated with **7a** and the solution was irradiated with u.v. light of 350 nm, the protein was radiolabelled with an efficiency of 5%. SDS-PAGE of the labelled protein as well as the radioautography showed that, as before[2], both the light and heavy chains were labelled. There was significantly higher incorporation of radioactivity (16%) with **5a** than **7a** (5%). Also, **5a** labelled only the heavy chain as shown by SDS-PAGE (Fig. 3). In the presence of a 30-fold excess of $MeGal_4$, the ligand

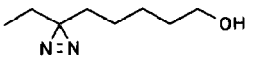


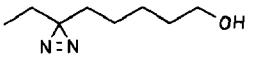
13 R′,R′ = R″,R‴ = Ip, R = 

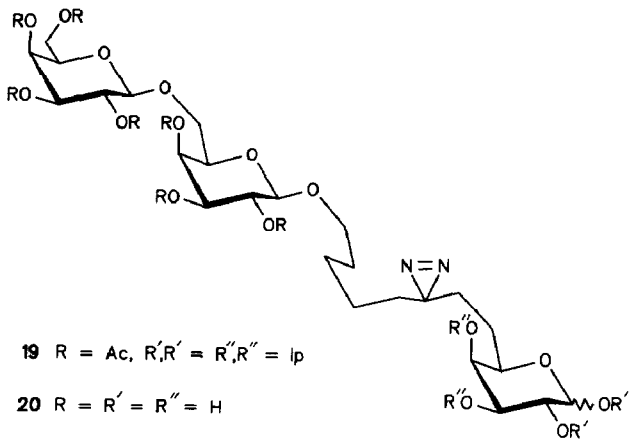
14 R′,R′ = R″,R‴ = Ip, R = 

15 R′,R′ = R″,R‴ = Ip, R = 

16 R′,R′ = R″,R‴ = Ip, R = 

17 R′,R′ = R″,R‴ = Ip, R = 

18 R′ = R″ = H, R = 



19 R = Ac, R′,R′ = R″,R‴ = Ip

20 R = R′ = R″ = H

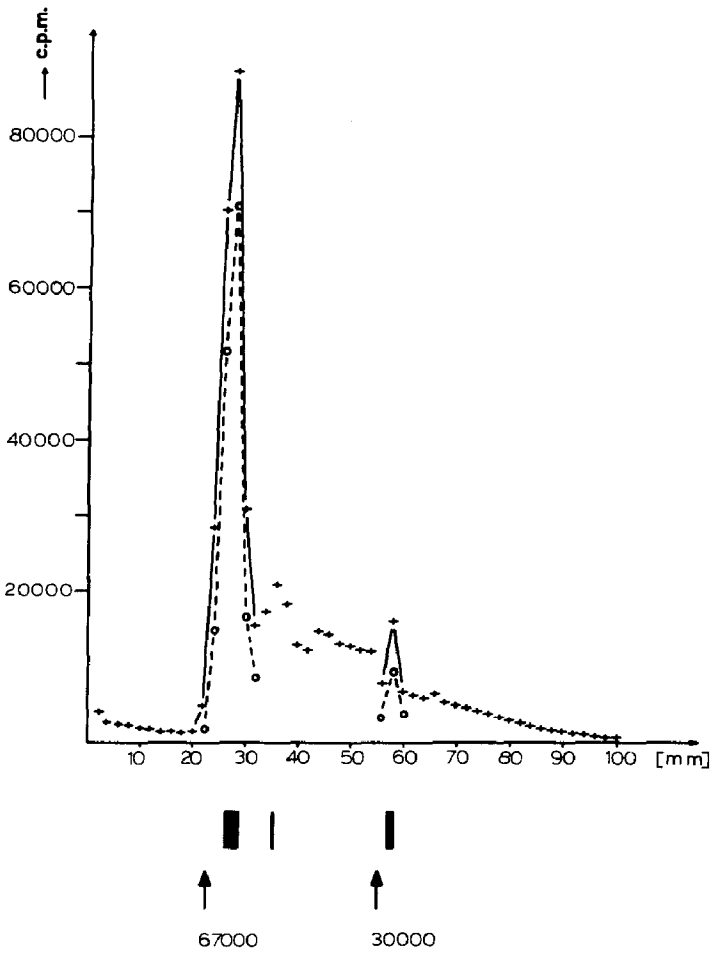


Fig. 3. SDS-PAGE of IgA J 539 after u.v.-light-induced labelling with **5a**. Significant incorporation of radioactivity was found only in the H-chain (- + -) and protection of **5a** by Gal_4-OMe (see Table I) was weak (-o-).

with the best known affinity for IgA X 24 and IgA J 539, labelling by **7a** was suppressed totally (Fig. 4), whereas that by **5a** was inhibited only to 4% (Fig. 3). When **5** was used as a competitive inhibitor for the protection of IgA J 539 against **7a**, the effect was the same as that obtained with Gal_4-OMe (Fig. 5).

DISCUSSION

The spacer-modified trisaccharide **20** (6-membered spacer) had slightly less affinity for IgA J 539 than **7** (4-membered spacer). This finding is not surprising since the additional freedom of movement increases the entropy and lowers the binding energy. Therefore, **7** appears to be ideally suited as a spacer-modified trisaccharide for photoaffinity labelling.

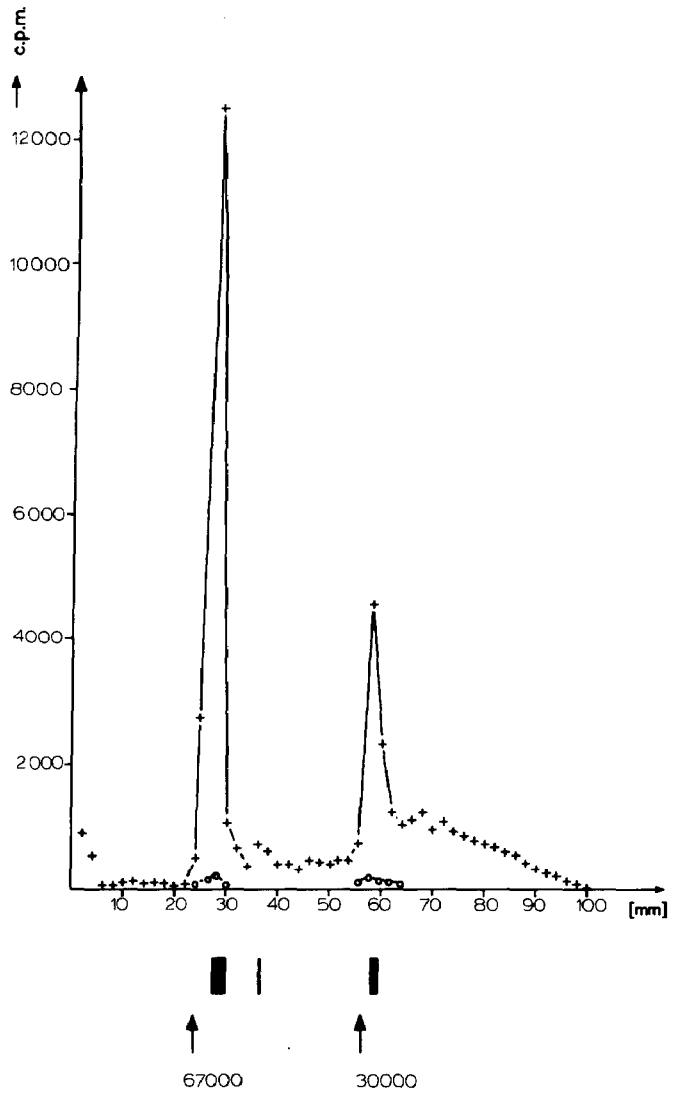


Fig. 4. SDS-PAGE of IgA J 539 after u.v.-light-induced labelling with **7a**. Incorporation of radioactivity is found in both the H- and L-chains (- + -). In the presence of Gal_4-OMe (see Table I), no radioactivity could be found (-o-).

The exchange of oxygen for sulfur as a bridging atom between the disaccharide moiety and the spacer in **12** does not alter the affinity significantly. The lower binding energy of **12** compared to that of **7** may be attributed to polar and/or steric factors.

Since **7** binds >6-times better than Gal_2-OMe (Table I), it is assumed that the disaccharide moiety of **7** does not occupy the two best binding subsites (A and B; binding mode I) which leaves the remainder of the molecule practically unbound. It is more likely that all three Gal*p* units are accommodated (binding mode II), which would mean that binding to subsites C, A, and D is better than to A and B.

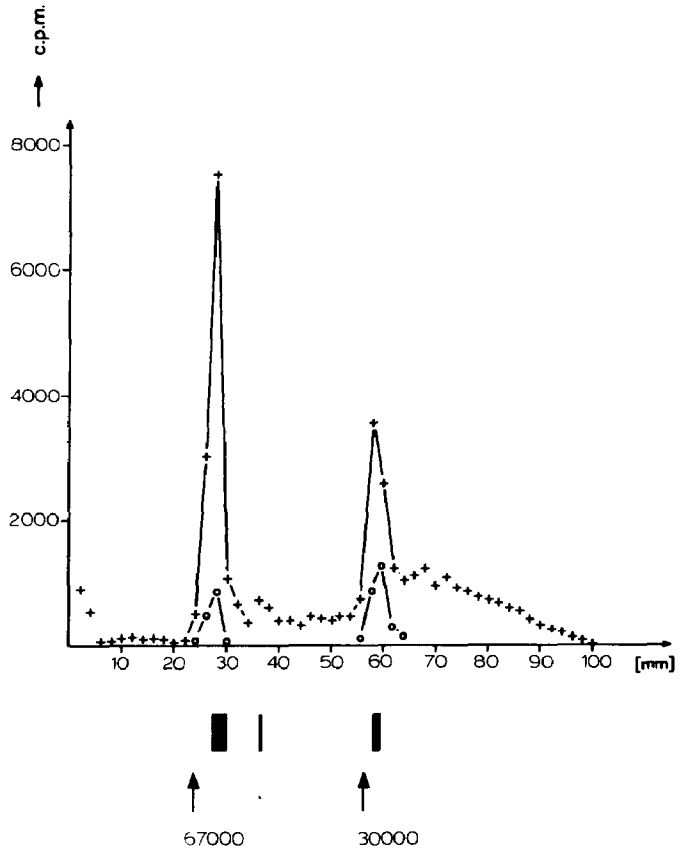


Fig. 5. SDS-PAGE of IgA J 539 after u.v.-light-induced labelling with **7a**. Incorporation of radioactivity is found in both the H- and L-chains (-+-). In the presence of **5**, hardly any radioactivity was incorporated (-o-).

Photoaffinity labelling by **7a** shows the same pattern as those[2,3] effected by the mono- (**1**) and di-saccharide (**2**) derivatives. In contrast to the smaller ligands **1** and **2**, **7** can be assumed to bind almost exclusively in mode II (Fig. 2) with the spacer in the area around binding site B. The labelling of both the heavy and the light chains in IgA J 539 by **7a** corroborates earlier proposals that these peptide chains meet in the area between subsites B and D since, considering mode II, the photolabile group is closer to binding site D than to binding site A.

As found in other investigations[10], a single Gal*p* unit should bind almost exclusively to subsite A since this would have the highest binding energy. This conclusion explains why **5**, which has the photolabile group adjacent to the glyconic side of the galactose moiety, will chemically modify parts of the binding area of the antibody different from those effected by all of the other photolabile ligands, including the *β*-galactoside **1** and the pseudo-disaccharide **2**. The pattern of labelling effected by **5a** (Fig. 3) shows no modification of the light chain. For binding mode III (Fig. 2) for **5a**, it

is assumed that labelling takes place in subsite C, which appears to be formed exclusively by the heavy chain.

Whereas labelling by **7**, as expected, can be prevented by a 30-fold molar excess of Gal_4-OMe, it has only a minor protective effect on labelling by compound **5a**, even though there is a great difference in affinities between **5** on the one hand and **7**, Gal_4, and Gal_4-OMe on the other. A similar apparent paradox involves the fact that **5a** is more efficient by a factor of 3.2 in labelling the antibody than is compound **7a**, although the latter has a much higher affinity.

These apparently contradictory and experimental findings can be explained if it is accepted that **5a** has a higher or comparable affinity for the binding site of the antibody than the oligomeric ligands.

In considering the way the binding constants are measured[9] and the different binding modes, it is clear that all ligands which occupy subsite A with extensions into subsite B and beyond, where the two indicator tryptophanyl moieties are located, cause a significant change in fluorescence whereby the degree of binding is monitored. Compound **5**, however, having its spacer attached to C-6 of the Gal*p* unit will not perturb the indicator significantly when the Gal*p* unit is in subsite A (Fig. 2, III). Therefore, a much higher concentration of **5** is needed than of the other ligands in order to cause the same fluorescence effect. Thus, the binding constant measured is probably not the real one.

The fact that **5** can protect the antibody effectively against labelling by **7a**, although it has an apparent affinity 2×10^3 lower than that of **7**, is evidence for a much higher real affinity of **5** than that actually measured by the fluorescence method. However, there is no doubt about the regioselectivity of labelling, determined by the photoreactive diazirino group attached in relation to the anchoring galactosyl unit in subsite A.

EXPERIMENTAL

General methods. — All reactions were monitored by t.l.c. on Silica Gel 60 F_{254} (Merck) and column chromatography was performed on Silica 32–63, 60 A (ICN). H.p.l.c. involved an LKB 2152 controller, two LKB 2150 pumps, a Rheodyne 7126 injector, an LKB variable wavelength monitor, and a Shimadzu C-R2Ax integrator. Preparative h.p.l.c. involved three Knauer 64 pumps, a dynamic mixing chamber, an injection valve, and a variable wavelength monitor. Columns (Bischoff) were used as indicated. Fluorescence measurements were obtained with a Perkin–Elmer 165/10s spectrometer, optical rotations with a Schmidt & Haensch Polartronic I polarimeter, and u.v. spectra and extinction coefficients with a Zeiss PMQ II spectrophotometer. ^{1}H-N.m.r. spectra (250 MHz) were recorded with a Bruker WM 250 spectrometer for solutions in $CDCl_3$ (internal Me_4Si). Melting points are uncorrected. Photolyses were performed with a Rayonet RPR 100 reactor equipped with 16 RPR 3500 A lamps. Radioactive material was detected with a Berthold Automatic TLC-Linear Analyzer, or by autoradiography using "Curix" X-ray film (Agfa-Gevaert), and assayed with a

Berthold BF 815 liquid scintillation counter, using Quickszint 501 (Zinsser) for solutions in organic solvents, and Quickszint 1 for aqueous solutions.

8-Azi-6,7,8,9,10-pentadeoxy-1,2:3,4-di-O-*isopropylidene*-α-D-galacto-*undecodialdo-1,5-pyranose* (**4**). — To a solution of 8-azi-6,7,8,9,10-pentadeoxy-1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-undecopyranose[5] (**3**; 77.3 mg, 0.22 mmol) in dry dichloromethane (30 mL) containing pyridine (200 μL) was added pyridinium chlorochromate (70 mg, 0.33 mmol). The mixture was stirred overnight and then concentrated *in vacuo*. The dark residue was dissolved in dichloromethane (20 mL) and eluted from a column of silica gel with 1:3 EtOAc–cyclohexane to give **4** (60.7 mg, 79%), isolated as a colourless syrup, $[\alpha]_D^{23}$ −41° (*c* 1.45, chloroform); λ_{max} 350 nm (ε_{mM} 50); R_F 0.56 (1:1 EtOAc–cyclohexane); ν_{max}^{film} 1735 cm^{-1} (C=O). ^{1}H-N.m.r. data ($CDCl_3$): δ 9.72 (t, 1 H, CHO), 5.51 (d, 1 H, $J_{1,2}$ 4.8 Hz, H-1), 4.58 (dd, 1 H, $J_{2,3}$ 2.7, $J_{3,4}$ 7.97 Hz, H-3), 4.29 (dd, 1 H, H-2), 4.06 (dd, 1 H, $J_{4,5}$ 1.65 Hz, H-4), 3.62 (m, 1 H, H-5), 2.28 (m, 2 H, $J_{10,11}$ 1.05, $J_{9,10}$ 7.8 Hz, H-10), 1.78 (dt, 2 H, CH_2), 1.73–1.21 (m, 4 H, 2 CH_2), 1.53 (s, 3 H, CH_3), 1.45 (s, 3 H, CH_3), 1.32 (d, 6 H, 2 CH_3).

8-Azi-6,7,8,9,10-pentadeoxy-1,2:3,4-di-O-*isopropylidene*-α-D-*(11-^{3}H)*galacto-*undecopyranose* (**3a**). — A solution of **4** (60.7 mg, 0.17 mmol) in 2:1 1,4-dioxane–water (1.4 mL) was treated with 0.5M NaOH (50 μL) and added to NaB^3H_4 (100 mCi, 7 $Ci.mmol^{-1}$). After 24 h, the mixture was concentrated *in vacuo*, and the residue was dissolved in the minimum amount of 1:1 EtOAc–cyclohexane and eluted from a column (1.5 × 20 cm) of silica gel with the same solvent to yield **3a** (45 mCi, 1.75 $Ci.mmol^{-1}$) that co-chromatographed [2D-t.l.c.: R_F 0.25 (1:1 EtOAc–cyclohexane) and 0.2 (1:10 EtOH–toluene)] with **3** as shown when the radioactivity was located by autoradiography.

8-Azi-6,7,8,9,10-pentadeoxy-D-*(11-^{3}H)*galacto-*undecose* (**5a**). — To a solution of **3a** (30 mCi, 1.75 $Ci.mmol^{-1}$; 6.1 mg, 17.14 μmol) in dichloromethane (2 mL) were added trifluoroacetic acid (1 mL) and water (50 μL). The mixture was left overnight at room temperature, then concentrated to dryness. The residue was dissolved in MeOH (2 mL), methanolic M sodium methoxide was added until a basic reaction persisted, and the mixture was left for 2 h. The solvent was evaporated under diminished pressure, and the residue was dissolved in water and subjected to h.p.l.c. (Spherisorb SS ODS 5 μm; column, 4.6 × 250 mm; 20:80 methanol–water, 0.4 mL/min) to give **5a** (20 mCi, 67%), which co-chromatographed [2D-t.l.c.: R_F α 0.25, β 0.31 (11:2:1 EtOAc–MeOH–water), and 0.15 (1:1 EtOH–toluene)] with **5** as shown when the radioactivity was located by autoradiography.

O-*(2,3,4,6-Tetra*-O-*acetyl*-β-D-*galactopyranosyl)*-*(1→6)*-O-*(2,3,4-tri*-O-*acetyl*-β-D-*galactopyranosyl)*-*(1→11)*-*8-azi-6,7,8,9,10-pentadeoxy-1,2:3,4-di*-O-*isopropylidene*-α-D-*(11-^{3}H)*galacto-*undecopyranose* (**6a**). — To a solution of **3a** (15 mCi, 1.75 $Ci.mmol^{-1}$) in dry dichloromethane (2 mL) were added silver silicate–alumina catalyst (0.5 g) and anhydrous $CaSO_4$ (20 mg), and the mixture was ultrasonicated for 1 h. To the stirred, cooled (−20°) suspension was added a solution of 2,3,4-tri-*O*-acetyl-6-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-α-D-galactopyranosyl bromide[4] (**8**; 20 mg, 29 μmol) in dichloromethane (1 mL) dropwise over 1 h. The mixture was allowed to attain

room temperature overnight, then filtered, and concentrated. Column chromatography (1:1 EtOAc–cyclohexane) of the residue gave **6a** (12 mCi, 80%) that co-chromatographed [2D-t.l.c.:R_F 0.19 (1:1 EtOAc–cyclohexane) and 0.14 (1:1 EtOH–toluene)] with **6** as shown when the radioactivity was located by autoradiography.

*O-β-D-Galactopyranosyl-(1→6)-O-β-D-galactopyranosyl-(1→11)-8-azi-6,7,8,9,10-pentadeoxy-D-(11-^{3}H)*galacto-*undecose* (**7a**). — Compound **6a** (12 mCi, 1.75 Ci.mmol^{-1}) was treated with trifluoroacetic acid as described for **5a**. H.p.l.c. (Spherisorb SS ODS 5 μm; column, 4.6 × 250 mm; 13:87 methanol–water, 0.4 mL/min) of the product gave **7a** (8 mCi, 61%) that co-chromatographed [2D-t.l.c.: R_F 0.26 (4:2:1 EtOAc–MeOH–water) and 0.37 (7:3:3:2:3:2 1-propanol–EtOH–EtOAc–pyridine–water–AcOH)] with **7** as shown when the radioactivity was located by autoradiography.

Reaction with IgA J 539. — (a) **5a** *and* **7a**. — Compounds **7a** (4 μCi, 22.39 × 10^{-9} mol) and **5a** (11.25 mCi, 0.71 × 10^{-6} mol) were each dissolved in a solution of antigalactan IgA J 539 (300 μL, A_{280} 2.22) in phosphate-buffered saline (pH 7.4) (solutions A and A′). One part (150 μL) of solutions A and A′ was made 2 × 10^{-3}M in Gal_4-OMe to give solutions B and B′. Solutions A, A′, B, and B′ were each deoxygenated with a stream of nitrogen, irradiated at 300–350 nm for 15 min, then dialysed against phosphate-buffered saline (pH 7.4) (3 ×) and water, and lyophilised, and the product was subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with Serva Blue, then cut into 2-mm slices, each of which was submerged overnight in Biolute-S (Zinsser, 0.5 mL). Quickszint-501 (4 mL, Zinsser) was then added, the mixture was kept for 2 h in the cold, and the radioactivity was determined.

(b) **7a** *in the presence of* **5**. — Compound **7a** (4 μCi, 22.39 × 10^{-9} mol) was dissolved in a solution of antigalactan IgA J 539 (300 μL, A_{280} 2.22) in phosphate-buffered saline (pH 7.4) (solution A). One part (150 μL) of solution A was made 20 × 10^{-3}M in **5** (solution B), and solutions A and B were treated as described in (*a*).

O-(2,3,4,6-Tetra-O-acetyl-β-D-galactopyranosyl)-(1→6)-2,3,4-tri-O-acetyl-1-S-benzoyl-1-thio-β-D-galactopyranose (**9**). — To a solution of **8** (2 g, 2.86 mmol) in dry acetone was added potassium thiobenzoate (1 g, 5.7 mmol). The mixture was stirred at room temperature for 2 h, then concentrated. Column chromatography (1:1 EtOAc–cyclohexane) of the residue gave amorphous **9** (2 g, 95%), R_F 0.47 (2:1 EtOAc–cyclohexane), $[\alpha]_D^{23}$ + 3° (*c* 0.96, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 8.01–7.45 (m, 5 H, Ph), 5.56–5.12 (m, 4 H, H-1,2,3,4), 5.34 (dd, 1 H, $J_{3',4'}$ 3.15, $J_{4',5'}$ 1.35 Hz, H-4′), 5.18 (dd, 1 H, $J_{1',2'}$ 8.25, $J_{2',3'}$ 10.5 Hz, H-2′), 4.98 (dd, 1 H, H-3′), 4.54 (d, 1 H, H-1′β), 4.18–4.05 (m, 3 H, H-5,6,6) 3.88–3.82 (m, 1 H, $J_{5',6'}$ 6.0 Hz, H-5′), 3.78 (d, 2 H, H-6′,6′), 2.2–1.96 (m, 21 H, 7 OAc);

Anal. Calc. for $C_{33}H_{40}O_{18}S$: C, 52.41; H, 5.29; S, 4.24. Found: C, 52.71; H, 5.30; S 4.28.

8-Azi-6,7,8,9,10-pentadeoxy-1,2:3,4-di-O-isopropylidene-11-O-tosyl-α-D-galacto-undecopyranose (**10**). — To a solution of **3** (600 mg, 1.68 mmol) in dry pyridine (25 mL) was added *p*-toluenesulfonyl chloride (642 mg, 3.37 mmol), and the mixture was kept at room temperature overnight. Excess of reagent was then hydrolysed with ice (5

g). After 1 h, the solution was poured into water (100 mL) and then, after 2 h, extracted with dichloromethane (4 × 30 mL). The combined extracts were neutralised with saturated aqueous $NaHCO_3$ (50 mL), washed with water (50 mL), dried (Na_2SO_4), and concentrated. Column chromatography (1:5 EtOAc–cyclohexane) of the residue gave **10** (663 mg, 75%), isolated as a slightly yellow syrup, $[\alpha]_D^{23}$ − 30° (*c* 0.84, chloroform); λ_{max} 350 nm (ε_{mM} 64.29); R_F 0.26 (1:3 EtOAc–cyclohexane). ^{1}H-N.m.r. data ($CDCl_3$): δ 7.78 (d, 2 H, ArH), 7.37 (d, 2 H, ArH), 5.53 (d, 1 H, $J_{1,2}$ 4.8 Hz, H-1), 4.58 (dd, 1 H, $J_{2,3}$ 2.7, $J_{3,4}$ 7.97 Hz, H-3), 4.29 (dd, 1 H, H-2), 4.12 (dd, 1 H, $J_{4,5}$ 1.65 Hz, H-4), 3.99–3.92 (m, 2 H, CH_2OTs), 3.72–3.65 (m, 3 H, CH_2O and H-5), 2.47 (s, 3 H, CH_3), 1.68–1.15 (m, 8 H, 4 CH_2), 1.52 (s, 3 H, CH_3), 1.48 (s, 3 H, CH_3), 1.33 (d, 6 H, 2 CH_3).

O-*(2,3,4,6-Tetra*-O-*acetyl-β*-D-*galactopyranosyl)-(1→6)*-S-*(2,3,4-tri*-O-*acetyl-β*-D-*galactopyranosyl)-(1→11)-8-azi-6,7,8,9,10-pentadeoxy-1,2:3,4-di*-O-*isopropylidene-11-thio-α*-D-galacto-*undecopyranose* (**11**). — To a solution of **9** (100 mg, 0.285 mmol) and **10** (215 mg, 0.285 mmol) in 1:1 acetone–water (4 mL) was added potassium carbonate (100 mg). The mixture was kept at 60° overnight, then concentrated *in vacuo*. Toluene (2 × 5 mL) was distilled from the residue, a solution of which in 3:1 pyridine–Ac_2O (5 mL) was kept at 40° for 2 h and then concentrated. Column chromatography (1:2 EtOAc–cyclohexane) of the residue gave amorphous **11** (158 mg, 56%), R_F 0.19 (1:1 EtOAc–cyclohexane), $[\alpha]_D^{23}$ − 19° (*c* 0.98, chloroform). ^{1}H-N.m.r. data ($CDCl_3$): δ 5.49 (d, 1 H, $J_{1,2}$ 4.8 Hz, H-1), 5.42–5.38 (m, 2 H, H-2′,4′), 5.18 (dd, 1 H, $J_{2',3'}$ 7.5, $J_{3',4'}$ 3.15 Hz, H-3′), 5.14 (dd, 1 H, $J_{3'',4''}$ 3.15, $J_{4'',5''}$ 1.2 Hz, H-4″), 5.02 (dd, 1 H, $J_{1'',2''}$ 7.8, $J_{2'',3''}$ 7.65 Hz, H-2″), 4.96 (dd, 1 H, H-3″), 4.58 (dd, 1 H, $J_{2,3}$ 2.7, $J_{3,4}$ 7.97 Hz, H-3), 4.52 (d, 1 H, H-1″), 4.41 (d, 1 H, $J_{1',2'}$ 10.2 Hz, H-1′), 4.29 (dd, 1 H, H-2), 4.17 (dd, 1 H, $J_{5'',6''b}$ 6.0, $J_{6''a,6''b}$ 11.25 Hz, H-6″b), 4.13 (dd, 1 H, $J_{5'',6''a}$ 6.0 Hz, H-6″a), 4.05 (dd, 1 H, $J_{4,5}$ 1.8 Hz, H-4), 3.91 (t, 1 H, H-5″), 3.88 (dd, 1 H, $J_{5',6'b}$ 6.0, $J_{6'a,6'b}$ 11.25 Hz, H-6′b), 3.83 (dd, 1 H, $J_{5',6'a}$ 6.0 Hz, H-6′a), 3.8–3.73 (m, 1 H, H-5′), 3.65–3.56 (m, 1 H, H-5), 2.78–2.51 (m, 2 H, H-11,11), 2.28–1.95 (m, 21 H, 7 OAc), 1.75–1.23 (m, 20 H, 4 CH_2 and 2 CMe_2).

Anal. Calc. for $C_{43}H_{62}N_2O_{22}S$: C, 52.15; H, 6.26; N, 2.83; S, 3.24. Found: C, 52.18; H, 6.21; N, 2.90; S, 3.21.

O-*β*-D-*Galactopyranosyl-(1→6)*-S-*β*-D-*galactopyranosyl-(1→11)-8-azi-6,7,8,9,10-pentadeoxy-11-thio*-D-galacto-*undecose* (**12**). — Compound **11** (100 mg, 0.1 mmol) was treated with trifluoroacetic acid as described for **5a**. H.p.l.c. (Hypersil ODS 5 μm; column; 20 × 250 mm; 12:88 methanol–water, 20 mL/min) of the product gave amorphous **12** (52 mg, 84%), $[\alpha]_D^{23}$ + 3.5° (*c* 1.4, water); λ_{max} 350 nm (ε_{mM} 25).

1-Benzyloxy-5-bromopentane. — Conventional treatment of 5-benzyloxypentanol (56 g, 0.29 mol) in dry pyridine (200 mL) with *p*-toluenesulfonyl chloride (77 g, 0.4 mol) at 0°, followed by column chromatography (1:8 EtOAc–cyclohexane) of the product, gave the 5-tosylate (75.3 g, 75%), isolated as a colourless syrup, R_F 0.31 (1:5 EtOAc–cyclohexane). ^{1}H-N.m.r. data ($CDCl_3$): 7.78 (d, 2 H, ArH), 7.30 (d, 2 H, ArH), 7.32 (m, 5 H, ArH), 4.45 (s, 2 H, OCH_2), 4.0 (t, 2 H, CH_2O), 3.4 (t, 2 H, CH_2O), 2.39 (s, 3 H, CH_3), 1.32–1.72 (m, 6 H, 3 CH_2).

A suspension of the 5-tosylate (70 g, 0.2 mol) and anhydrous lithium bromide (43.6 g, 0.5 mol) in acetone (800 mL) was boiled under reflux for 2 h, then filtered, and

concentrated *in vacuo*. A solution of the residue in dichloromethane (200 mL) was washed with water (2 × 100 mL), then saturated aqueous $NaHCO_3$ (100 mL), dried (Na_2SO_4), and concentrated. The colourless residue was distilled to give the title compound (30.8 g, 60%), b.p. 135°/0.1 Torr, as a colourless oil, R_F 0.53 (1:5 EtOAc–cyclohexane). ^{1}H-N.m.r. data ($CDCl_3$): δ 7.21 (d, 2 H, ArH), 4.42 (s, 2 H, OCH_2), 3.49–3.18 (m, 4 H, CH_2O and CH_2Br), 1.98–1.29 (m, 6 H, 3 CH_2).

Anal. Calc. for $C_{12}H_{17}BrO$: C, 56.07; H, 6.61; Br, 31.09. Found: C, 55.84; H, 6.53; Br, 30.80.

13-O-*Benzyl-6,7,9,10,11,12-hexadeoxy-1,2:3,4-di*-O-*isopropylidene*-DL-glycero-α-D-galacto-*tridecopyranose* (**14**). — To a suspension of 1-benzyloxy-pent-5-ylmagnesium bromide, prepared from Mg (2.9 g, 0.12 mol) and the foregoing bromide (15 g, 59 mmol) in dry ether (100 mL), was added a solution of 6,7-dideoxy-1,2:3,4-di-*O*-isopropylidene-α-D-*galacto*-octodialdo-1,5-pyranose[5] (**13**; 8.4 g, 29.5 mmol) in ether (50 mL) dropwise during 1 h. The mixture was stirred for 15 min, poured on to ice, and treated with saturated aqueous NH_4Cl (750 mL). The mixture was extracted with ether (4 × 100 mL), and the combined extracts were washed with water (100 mL), dried (Na_2SO_4), and concentrated *in vacuo*. Column chromatography (1:3 EtOAc–cyclohexane) of the residue gave **14**, isolated as a colourless syrup (14.9 g, 92%), R_F 0.17 (1:2 EtOAc–cyclohexane). ^{1}H-N.m.r. data ($CDCl_3$): δ 7.32 (m, 5 H, Ph), 5.53 (d, 1 H, $J_{1,2}$ 4.8 Hz, H-1), 4.58 (dd, 1 H, $J_{2,3}$ 2.7, $J_{3,4}$ 7.97 Hz, H-3), 4.5 (s, 2 H, OCH_2), 4.29 (dd, 1 H, H-2), 4.12 (dd, 1 H, $J_{4,5}$ 1.65 Hz, H-4), 3.75 (m, 1 H, H-5), 3.62 (m, 1 H, H-8), 3.47 (t, 2 H, OCH_2), 1.96 (s, 1 H, OH), 1.48–1.73 (m, 12 H, 6 CH_2), 1.52 (s, 3 H, CH_3), 1.48 (s, 3 H, CH_3), 1.33 (d, 6 H, 2 CH_3).

Anal. Calc. for $C_{26}H_{40}O_7$: C, 67.27; H, 8.62. Found: C, 66.99; H, 8.83.

13-O-*Benzyl-6,7,9,10,11,12-hexadeoxy-1,2:3,4-di*-O-*isopropylidene*-α-D-galacto-*tridecopyranos-8-ulose* (**15**). — To a solution of **14** (8 g, 17.2 mmol) in dry dichloromethane (250 mL) containing pyridine (1 mL) was added pyridinium chlorochromate (5.6 g, 25.8 mmol). The mixture was stirred overnight and then concentrated *in vacuo*. The dark residue was dissolved in dichloromethane (20 mL) and eluted from a column of silica gel with 1:3 EtOAc–cyclohexane. Recrystallisation from ethanol–water yielded **15** as needles (5.9 g, 74%), m.p. 46°, $[\alpha]_D^{23}$ +29° (*c* 1.1, chloroform); R_F 0.15 (1:5 EtOAc–cyclohexane); ν_{max}^{KBr} 1700 cm^{-1} (C = O). ^{1}H-N.m.r. data ($CDCl_3$): δ 7.31 (m, 5 H, Ph), 5.51 (d, 1 H, $J_{1,2}$ 4.8 Hz, H-1), 4.58 (dd, 1 H, $J_{2,3}$ 2.7, $J_{3,4}$ 7.97 Hz, H-3), 4.5 (s, 2 H, OCH_2), 4.29 (dd, 1 H, H-2), 4.12 (dd, 1 H, $J_{4,5}$ 1.65 Hz, H-4), 3.73 (m, 1 H, H-5), 3.46 (t, 2 H, CH_2O), 2.56 (m, 2 H, H-7,7), 2.42 (t, 2 H, H-9,9), 1.87 (m, 2 H, H-10,10), 1.75–1.51 (m, 6 H, 3 CH_2), 1.52 (s, 3 H, CH_3), 1.48 (s, 3 H, CH_3), 1.33 (d, 6 H, 2 CH_3).

Anal. Calc. for $C_{26}H_{38}O_7$: C, 67.56; H, 8.22. Found: C, 67.30; H, 8.02.

8-Azi-13-O-*benzyl-6,7,8,9,10,11,12-heptadeoxy-1,2:3,4-di*-O-*isopropylidene*-α-D-galacto-*tridecopyranose* (**16**). — Ammonia (30 mL) was condensed at −50° into a solution of **15** (3 g, 6.5 mmol) in dry methanol (100 mL). After 2 h, hydroxylamine-*O*-sulfonic acid (0.95 g, 8.4 mmol) was added portionwise to the vigorously stirred mixture during 1 h. The solution was then allowed to attain room temperature overnight, filtered, concentrated *in vacuo* to half of the original volume, and co-concentrated with

methanol (50 mL) to 50 mL. To the cooled (0°) solution was added Et_3N (2 mL) together with iodine until the red colour persisted for 30 min at room temperature. The mixture was concentrated, and a solution of the residue in chloroform (50 mL) was washed with dilute aqueous sodium thiosulfate and water, dried (Na_2SO_4), and concentrated under diminished pressure. Column chromatography (1:9 EtOAc–cyclohexane) of the residue gave **16**, isolated as a colourless syrup (1.7 g, 54%), $[\alpha]_D^{23}$ +12° (*c* 1.0, chloroform); λ_{max} 350 nm (ε_{mM} 49); R_F 0.23 (8:1 EtOAc–cyclohexane). ^{1}H-N.m.r. data ($CDCl_3$): δ 7.37 (m, 5 H, Ph), 5.53 (d, 1 H, $J_{1,2}$ 4.8 Hz, H-1), 4.58 (dd, 1 H, $J_{2,3}$ 2.7, $J_{3,4}$ 7.97 Hz, H-3), 4.48 (s, 2 H, OCH_2), 4.29 (dd, 1 H, H-2), 4.05 (dd, 1 H, $J_{4,5}$ 1.65 Hz, H-4), 3.62 (m, 1 H, H-5), 3.42 (t, 2 H, CH_2O), 1.68–1.01 (m, 12 H, 6 CH_2), 1.52 (s, 3 H, CH_3), 1.48 (s, 3 H, CH_3), 1.33 (d, 6 H, CH_3).

Anal. Calc. for $C_{26}H_{38}N_2O_6$: C, 65.85; H, 8.01. Found: C, 64.59; H, 7.90.

*8-Azi-6,7,8,9,10,11,12-heptadeoxy-1,2:3,4-di-*O*-isopropylidene-α-*D*-galacto-tridecopyranose* (**17**). — To a vigorously stirred suspension of potassium carbonate (1 g) in dry benzene (150 mL) were added **16** (1.7 g, 3.6 mmol) and bromine (0.57 g, 7.2 mmol) followed by irradiation with a commercial 100 VA lamp for 15 min at room temperature. The mixture was poured into water and extracted with chloroform (4 × 50 mL), and the combined extracts were washed with dilute aqueous sodium thiosulfate (100 mL) and water (100 mL), dried (Na_2SO_4), and concentrated. Column chromatography (1:2 EtOAc–cyclohexane) of the residue gave **17** (0.85 g, 62%), isolated as a slightly yellow syrup, $[\alpha]_D^{23}$ +22° (*c* 1.0, methanol); λ_{max} 350 nm (ε_{mM} 49); R_F 0.26 (1:1 EtOAc–cyclohexane); ν_{max}^{film} 3490 cm^{-1} (OH). ^{1}H-N.m.r. data ($CDCl_3$): δ 5.52 (d, 1 H, $J_{1,2}$ 4.8 Hz, H-1), 4.59 (dd, 1 H, $J_{2,3}$ 2.7, $J_{3,4}$ 7.97 Hz, H-3), 4.31 (dd, 1 H, H-2), 4.05 (dd, 1 H, $J_{4,5}$ 1.65 Hz, H-4), 3.62 (m, 3 H, CH_2O and H-5), 1.73–1.07 (m, 12 H, 6 CH_2), 1.52 (s, 3 H, CH_3), 1.48 (s, 3 H, CH_3), 1.33 (d, 6 H, 2 CH_3).

*8-Azi-6,7,8,9,10,11,12-heptadeoxy-*D*-galacto-tridecose* (**18**). — To a solution of **17** (300 mg, 0.78 mmol) in chloroform (20 mL) was added trifluoroacetic acid (2 mL, 99%). The mixture was stored at room temperature for 24 h, then concentrated, and methanol was distilled from the residue, a solution of which in methanol (50 mL) was treated with methanolic M sodium methoxide until the mixture was alkaline. When the reaction was complete (R_F 0.16 α, 0.13 β; 11:2:1 EtOAc–MeOH–water), the solution was neutralised with Amberlite IR-120 (H^+) resin, filtered, and concentrated *in vacuo*. H.p.l.c. (Hypersil ODS 5 μm; column, 20 × 250 mm; 12:88 methanol–water, 20 mL/min) of the residue gave amorphous **18** (194.7 mg, 82%), $[\alpha]_D^{23}$ +51° (*c* 0.87, water), λ_{max} 350 nm (ε_{mM} 48).

Anal. Calc. for $C_{13}H_{24}N_2O_6$: C, 51.35; H, 7.83; N, 9.21. Found: C, 51.42; H, 7.87; N, 9.74.

*O-(2,3,4,6-Tetra-*O*-acetyl-β-*D*-galactopyranosyl)-(1→6)-*O*-(2,3,4-tri-*O*-acetyl-β-*D*-galactopyranosyl)-(1→13)-8-azi-6,7,8,9,10,11,12-heptadeoxy-1,2:3,4-di-*O*-isopropylidene-α-*D*-galacto-tridecopyranose* (**19**). — To a solution of **17** (330 mg, 0.86 mmol) in dry dichloromethane (20 mL) were added silver silicate–alumina catalyst (1.5 g), anhydrous $CaSO_4$ (0.7 g), and 3 Å molecular sieve (1 g), and the mixture was ultrasonicated for 1 h. To a stirred, cooled (−20°) suspension was added dropwise a solution of **8**

(698 mg, 1 mmol) in dichloromethane (5 mL) during 1 h. The mixture was allowed to attain room temperature overnight, then filtered, neutralised with saturated aqueous $NaHCO_3$ (20 mL), washed with water (20 mL), dried (Na_2SO_4), and concentrated. Column chromatography (1:1 EtOAc–cyclohexane) of the residue gave amorphous **19** (560 mg, 66%), $[\alpha]_D^{23}$ +17° (*c* 0.96, methanol); λ_{max} 350 nm (ε_{mM} 50); R_F 0.19 (1:1 EtOAc–cyclohexane). ^{1}H-N.m.r. data ($CDCl_3$): δ 5.49 (d, 1 H, $J_{1,2}$ 4.8 Hz, H-1), 5.42–5.38 (m, 2 H, H-2′,4′), 5.18 (dd, 1 H, $J_{2',3'}$ 7.5, $J_{3',4'}$ 3.15 Hz, H-3′), 5.14 (dd, 1 H, $J_{3'',4''}$ 3.15, $J_{4'',5''}$ 1.2 Hz, H-4″), 5.02 (dd, 1 H, $J_{1'',2''}$ 7.8, $J_{2'',3''}$ 7.65 Hz, H-2″), 4.96 (dd, 1 H, H-3″), 4.58 (dd, 1 H, $J_{2,3}$ 2.7, $J_{3,4}$ 7.97 Hz, H-3), 4.52 (d, 1 H, H-1″), 4.41 (d, 1 H, $J_{1',2'}$ 7.65 Hz, H-1′), 4.29 (dd, 1 H, H-2), 4.17 (dd, 1 H, $J_{5'',6''b}$ 6.0, $J_{6''a,6''b}$ 11.25 Hz, H-6″b), 4.13 (dd, 1 H, $J_{5'',6''a}$ 6.0 Hz, H-6″a), 4.05 (dd, 1 H, $J_{4,5}$ 1.8 Hz, H-4), 3.91 (t, 1 H, $J_{5'',6''}$ 6.0 Hz, H-5″), 3.87 (dd, 1 H, $J_{5',6'b}$ 6.0, $J_{6'a,6'b}$ 11.25 Hz, H-6′b), 3.77 (dd, 1 H, $J_{5',6'a}$ 6.0, H-6′a), 3.63–3.56 (m, 1 H, H-5), 4.91–3.5 (m, 1 H, H-5′), 2.2–1.96 (m, 21 H, 7 OAc), 1.75–1.23 (m, 26 H, 7 CH_2, 4 CH_3).

Anal. Calc. for $C_{45}H_{66}N_2O_{23}$: C, 53.93; H, 6.58; N, 2.79. Found: C, 53.41; H, 6.44; N, 2.82.

O-*β*-D-*Galactopyranosyl-(1→6)*-O-*β*-D-*galactopyranosyl)-(1→13)-8-azi-6,7,8,9,10,11,12-heptadeoxy*-D-galacto-*tridecose*. (**20**). — Compound **19** (560 mg, 0.56 mmol) was treated with trifluoroacetic acid, as described for **18**. H.p.l.c. (Hypersil ODS 5 μm; column, 20 × 250 mm; 35:65 methanol–water, 20 mL/min) of the product gave amorphous **20** (256.8 mg, 73%), R_F 0.16 (4:2:1 EtOAc–MeOH–water), $[\alpha]_D^{23}$ +12° (*c* 1.2, water; λ_{max} 350 nm (ε_{mM} 38).

Anal. Calc. for $C_{25}H_{44}N_2O_{16}$: C, 47.80; H, 7.00; N, 4.46. Found: C, 47.69; H, 7.12; N, 4.54.

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