The Properties of a "Synthetic" Antigen Related to the Human Blood-Group Lewis a

R. U. Lemieux,* D. R. Bundle,[1] and D. A. Baker[2]

Contribution from the Department of Chemistry, University of Alberta, Edmonton, Alberta, Canada T6G 2G2. *Received October 4, 1974*

Abstract: The β-glycosides of 2-acetamido-2-deoxy-D-glucopyranose, 2-acetamido-2-deoxy-4-*O*-(α-L-fucopyranosyl)-D-glucopyranose, 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-D-glucopyranose, and 2-acetamido-2-deoxy-4-*O*-(α-L-fucopyranosyl)-3-*O*-(β-D-galactopyranosyl)-D-glucopyranose were synthesized, using either 8-ethoxycarbonyl- or 8-methoxycarbonyl-1-octanol. Semisynthetic antigens were prepared from these esters by attachment to the free amino groups in bovine serum albumin. The antibodies raised with the trisaccharide antigen which possesses the terminal trisaccharide unit of the human blood-group Lewis a (Le^a) determinant precipitated natural blood-group Le^a substance and agglutinated Le^a red blood cells. Other immunochemical cross-reactions as well as inhibition experiments are reported.

The success achieved in the synthesis of oligosaccharide structures using the so-called oximinochloride[3,4] and halide ion[5-7] catalyzed glycosidation reactions augurs well for a capability to synthesize substantial portions of the oligosaccharide antigenic determinants of polysaccharide, glycoprotein, and glycolipid antigens. The most noteworthy achievements to date in this regard were the syntheses of 3-*O*-(2-acetamido-2-deoxy-α-D-galactopyranosyl)-D-galactopyranose (the terminal disaccharide unit of the human blood-group A determinant),[8] 2-*O*-(α-L-fucopyranosyl)-3-*O*-(α-D-galactopyranosyl)-D-galactose (the terminal trisaccharide unit of the human blood-group B determinant),[7] 2-*O*-(α-L-fucopyranosyl)-D-galactose (the terminal disaccharide unit of the human blood-group O(H) determinant),[7,9] and 2-acetamido-2-deoxy-4-*O*-(α-L-fucopyranosyl)-3-*O*-(β-D-galactopyranosyl)-D-glucose (the terminal trisaccharide unit of the human blood-group Le^a).[6] Methodology was established in the first instance for the synthesis of these reducing oligosaccharides primarily for the comparison of the synthetic products with those obtained by hydrolysis of the blood-group glycoproteins. However, the synthesis of reducing oligosaccharides per se is of limited value, and such syntheses have been largely discontinued in this laboratory. Instead, attention is now focused on the synthesis of the oligosaccharides in the form of glycosides that possess an aglycon which can serve as a bridge for the attachment of the oligosaccharide to high molecular weight substances. Thus, covalent attachment to proteins or other high polymers would provide antigens with which to study the immunochemistry and immunology of carbohydrate antigens. The immunochemical aspects of such developments based in chemical synthesis are considered particularly exciting since these would allow the study of chemically modified antigens in such a way as perhaps to shed important insight on the mechanism of the binding with the active sites of the antibody.

The use of synthetic oligosaccharides in a form ready for attachment to polymeric material is not restricted to the synthesis of immunogens but could conceivably be valuable for the preparation of immunoabsorbents for affinity chromatography and, also, for the preparation of target cells, such as red blood cells, for the study of immunological responses in suitably immunized animals. Indeed the work to date has substantiated the validity of all of these objectives.

The preparation of synthetic antigens from carbohydrates and proteins is not new.[10] The pioneering work of Goebel and Avery[11,12] showed that antibody specific for carbohydrate structures could be generated in experimental animals immunized with synthetic antigen. In many instances, however, these investigations employed coupling strategies which introduced antigenic sites in the determinant not present in the natural antigen. The method used by Goebel and Avery[11,13] involved the preparation of *p*-aminophenyl or *p*-aminobenzyl glycosides which, after diazotization, could be covalently linked to the protein.[12] The resulting immunogen raised a spectrum of antibodies in rabbits which were specific for the oligosaccharide and aromatic portions of the hapten as well as to the protein carrier.[12] The preparation of the *p*-aminophenyl or *p*-aminobenzyl glycosides requires the availability of the oligosaccharide, and therefore such investigations,[10] particularly those of Goldstein and coworkers,[14-16] are largely limited to available natural structures. Nevertheless, valuable information on problems of biological significance was achieved. The elegant studies of Lüderitz, Westphal, and Staub[17] utilized the technique of Goebel and Avery[11] for the attachment of monosaccharides related to the bacterial *O*-antigens. A variation of this method involves conversion of the amino group to isothiocyanate[18] instead of diazonium salt.

Rüde and coworkers[19] have synthesized serine glycosides of mono- and disaccharides and have coupled these to protein as the *O*-acetyl derivatives by way of the Leuchs-type anhydride.[20]

Much ingenuity has been involved in the establishment of methods for the attachment of sugars and oligosaccharides to carrier polymers. Kabat and coworkers[21] have oxidized the reducing saccharide to aldonic acid which was subsequently attached to protein amino groups by way of a mixed anhydride. Westphal and coworkers[22] converted the saccharide to 1-(*m*-nitrophenyl)flavazole and accomplished the coupling by reduction to amine followed by diazotization. This method requires that the 2- and 3-positions of the reducing unit of the saccharide be unsubstituted. Bishop and coworkers[23] have used cyanuric trichloride to couple polysaccharide to protein and more recently[24] have converted the reducing carbon of monosaccharides to amines and established a linkage between this amine, 2-amino-2-deoxy-D-hexoses, and the amino groups of glycine by reaction with cyanuric trichloride. These methods suffer in the case of low molecular weight oligosaccharides both because they destroy the terminal reducing unit of the saccharide and because they introduce important foreign structure to the hapten. However, as recently displayed by Westphal and coworkers,[25] these disadvantages are unimportant if the resulting hapten is large as is the case of a tetrasaccharide of bacterial origin.

Total synthesis of the hapten eliminates the problem of the availability of oligosaccharides. These often can only be

obtained from natural sources in minute amounts, with great difficulty and as reducing sugars.

Chemical synthesis of the complete hapten, as is reported in this paper, could have employed one of the above coupling techniques. However, in view of the antigenicity of aromatic structures,[14,15,26] it was decided to proceed instead by way of a long aliphatic chain. The choice of nine carbons was convenient and considered appropriate to minimize intramolecular reaction of the activated molecule, the 8′-azidocarbonyloctyl aglycon.

Results and Discussion

Ethyl 9-hydroxynonanoate (**2**) was prepared by diborane reduction[27] of the half ester of azelaic acid **1**. The 2-acetamido-2-deoxy-β-D-glucopyranoside **5** (see Chart I) of this alcohol was prepared under Koenigs–Knorr conditions, and the terminal trisaccharide unit; i.e., 2-acetamido-2-deoxy-4-*O*-(α-L-fucopyranosyl)-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranosyl, of the human blood-group Le^a [28] was developed (see Figure 1) from this substance to provide the potential hapten **36** following closely the procedures reported for the synthesis of the trisaccharide[6] and, therefore, description of this methodology is not repeated in detail. In addition to compounds **5** and **36**, the 8-methoxycarbonyloctyl glycosides of the two component disaccharides of **36** were prepared, namely, the 2-acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (**28**) and the 2-acetamido-2-deoxy-4-*O*-(α-L-fucopyranosyl)-β-D-glucopyranoside (**19**). Saponification to the sodium salt provided water-soluble products for nuclear magnetic resonance spectroscopy.

The ^{13}C NMR spectra in each case established the presence of the expected number of carbon atoms and required high purity. The assignments (see Table I) followed the usual procedure[6] and are in agreement with the findings made in connection with the reducing oligosaccharides. The most noteworthy features in these spectra are the unexpectedly high shieldings found for C-3 and C-4 of the *N*-acetyl-D-glucosamine residue of both the ester **36** and the salt **37.** The introduction of both the β-D-galactopyranosyl and α-L-fucopyranosyl groups appear to have caused deshielding of these carbons by only 2.2 and 2.1 ppm, respectively, in contrast to the more normal 8.6 ppm (**6** vs. **29**) and 7.4 ppm

Chart I

Compounds

$C_2H_5OCO(CH_2)_7COOH$

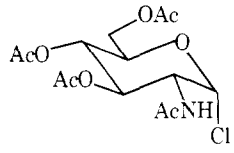
1

$C_2H_5OCO(CH_2)_7CH_2OH$

2

Definitions:
E = $C_2H_5OCO(CH_2)_8$
M = $CH_3OCO(CH_2)_8$
S = $COO^-(CH_2)_8$
Y = $H_2NNHCO(CH_2)_8$
Z = $N_3CO(CH_2)_8$
BSA = bovine serum albumin
n = degree of substitution
A = $\frac{BSA}{n}$ -$NHCO(CH_2)_8$

Ac = acetyl, Bn = benzyl, Bz = benzoyl



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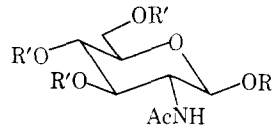
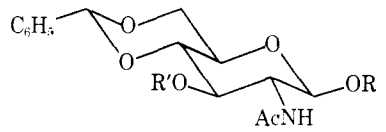
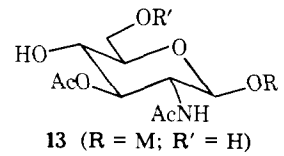


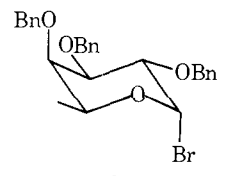
4 (R = E; R′ = Ac)
5 (R = E; R′ = H)
6 (R = S; R′ = H)
7 (R = Y; R′ = H)
8 (R = Z; R′ = H)
9 (R = A; R′ = H)
10 (R = M; R′ = H)



11 (R = M; R′ = H)
12 (R = M; R′ = Ac)
24 (R = E; R′ = H)



13 (R = M; R′ = H)
14 (R = M; R′ = Bz)



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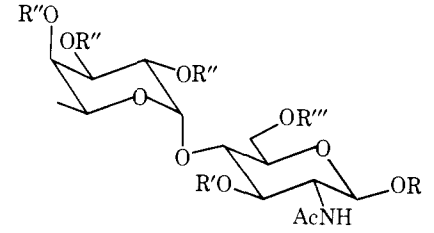
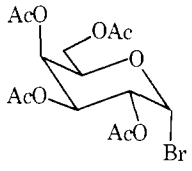


17 (R = M; R′ = Ac; R″ = Bn; R‴ = Bz)
18 (R = M; R′ = R‴ = H; R″ = Bn)
19 (R = M; R′ = R″ = R‴ = H)
20 (R = S; R′ = R″ = R‴ = H)
21 (R = Y; R′ = R″ = R‴ = H)
22 (R = Z; R′ = R″ = R‴ =H)
23 (R = A; R′ = R″ = R‴ = H)



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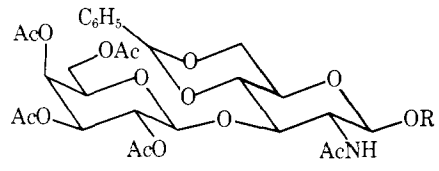
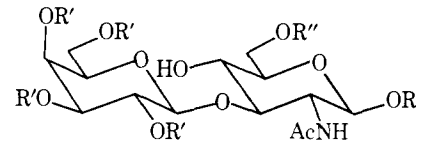
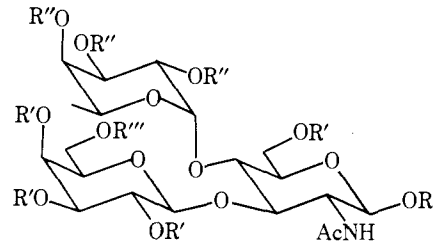


26 (R = E)



27 (R = E; R′ = Ac; R″ = H)
28 (R = E; R′ = R″ = H)
29 (R = S; R′ = R″ = H)
30 (R = Y; R′ = R″ = H)
31 (R = Z; R′ = R″ = H)
32 (R = A; R′ = R″ = H)
33 (R = E; R′ = R″ = Ac)



34 (R = E; R′ = R‴ = Ac; R″ = Bn)
35 (R = M; R′ = H; R″ = Bn; R‴ = Ac)
36 (R = M; R′ = R″ = R‴ = H)
37 (R = S; R′ = R″ = R‴ = H)
38 (R = Y; R′ = R″ = R‴ = H)
39 (R = Z; R′ = R″ = R‴ = H)
40 (R = A; R′ = R″ = R‴ = H)

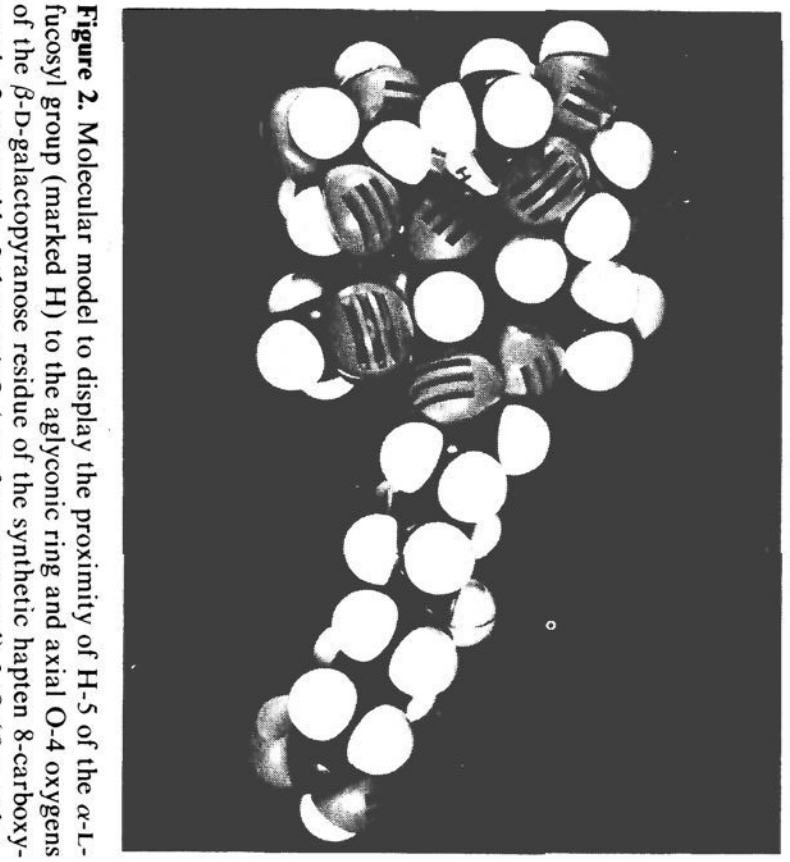


Figure 1. Outline of the synthetic sequences leading to the antigens **9**, **23**, **32**, and **40**.

Figure 2. Molecular model to display the proximity of H-5 of the α-L-fucosyl group (marked H) to the aglyconic ring and axial O-4 oxygens of the β-D-galactopyranose residue of the synthetic hapten 8-carboxyoctyl 2-acetamido-2-deoxy-4-*O*-(α-L-fucopyranosyl)-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside.

(**6** vs. **20**) downfield shifts when only one of the branching sugars was introduced. As will be seen below, H-5″ of the fucosyl group is strongly deshielded in the trisaccharide structures **36** and **37**. The phenomena responsible for these ^{1}H NMR shifts do not appear to have caused important shielding of C-5″ relative to the chemical shift of C-5 in methyl α-L-fucopyranoside.[6]

A goal of these synthetic studies was to obtain compounds that could serve as models for the study of the conformational properties of the blood-group antigenic determinants. The conformational properties of glycopyranoside structures have recently been reviewed,[29] and considerations based on nonbonded interactions as assessed by hard-sphere calculations and preferred bond orientation as the result of the exo anomeric effect were considered. Application of this methodology[30] to the Le^a determinant provided a conformation close to that represented by the model in Figure 2. It is seen that H-5″ is in very close proximity to O-3 (glucosamine residue), O-4′, and O-5′ (galactose residue). Thus, the abnormal chemical shifts for H-5″ noted in Table II can be appreciated in view of the electrostatic

Table I. ^{13}C-Nuclear Magnetic Resonance Chemical Shifts in Deuterium Oxide Relative to Tetramethylsilane in CCl_4 (1:1, ppm, external)

	N-Acetyl-β-D-glucosamine residue								β-D-Galactose residue						α-L-Fucose residue						Aglycon						
Compd	C-1	C-2	C-3	C-4	C-5	C-6	CO	CH_3	C-1	C-2	C-3	C-4	C-5	C-6	C-1	C-2	C-3	C-4	C-5	C-6	CO	CH_2	CH_2	$(CH_2)_4$	CH_2	CH_2O	OCH_3
6	101.4	55.9	74.1	70.3	76.1	61.1	174.6	22.6													184.1	37.9	25.4	28.9	26.2	70.7	
20	101.6	56.6	73.2	78.1	75.7	60.6	174.8	22.8							100.1	72.5	68.7	70.0	67.5	15.8	184.1	38.0	25.6	29.1	28.9	70.9	
29	101.4	55.1	83.1	69.1	75.8	61.5	174.9	22.9	104.0	71.2	73.0	69.1	75.8	61.5							184.2	38.1	25.6	29.1	26.4	71.0	
36	101.5	56.4	76.7	73.1	75.4	60.5	174.3	23.0	103.3	71.1	73.1	68.9	76.0	62.2	98.6	72.5	68.4	69.7	67.3	16.0	177.3	34.3	25.0	29.1	25.7	70.8	52.5
37	101.4	56.3	76.7	72.8	75.2	60.3	174.6	22.9	103.3	71.0	72.8	68.8	75.8	62.0	98.5	72.4	68.4	69.6	67.2	15.9	184.3	38.1	25.5	29.1	26.3	71.0	

Table II. Proton Magnetic Resonance Spectra in Deuterium Oxide

	Chemical shifts,[a] τ (spacing, Hz)				
	Residue				
	Gluco	Galacto	Fuco		
Compd	H-1	H-1′	H-1″	H-5″[b]	CH_3″
6	5.24 (8.0)				
20	5.30 (7.5)		4.86 (~2.0)	5.46[d] (6.5)	8.64 (6.5)
29	5.34 (7.0)	5.36[c]			
37	5.44 (7.0)	5.40 (7.0)	4.91 (2.5)	5.14 (6.5)	8.72 (6.5)

[a] Relative to tetramethylsilane in CCl_4 (1:1, external). [b] The coupling of H-5″ with H-4″ was weak ~1 Hz. [c] Appeared as multiplet, half-band width ~7 Hz. [d] The chemical shift of H-5 for methyl α-L-fucopyranoside is τ 5.93.

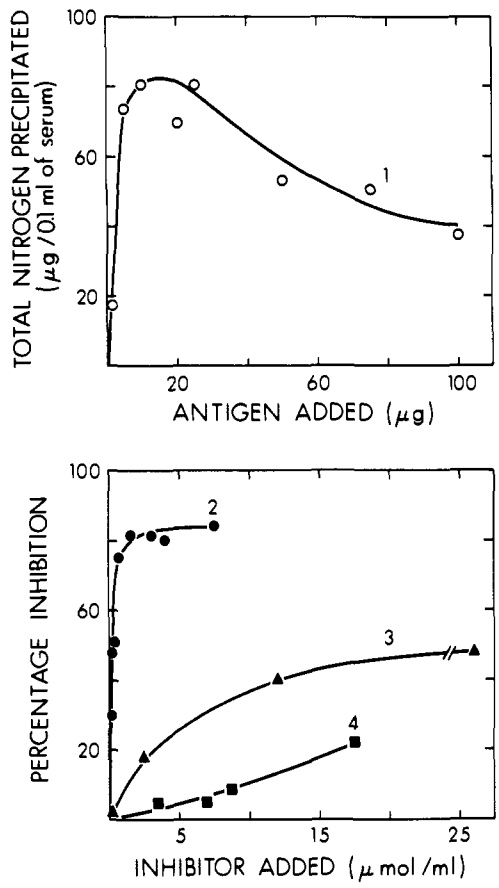


Figure 3. Precipitation and inhibition experiments using the serum of a rabbit immunized with the "synthetic" Le^a antigen. Plot 1, precipitation by the "synthetic" Le^a antigen. Plots 2, 3, and 4, inhibition of the precipitation in plot 1 by increasing amounts of the potassium salts of **37, 29,** and **20,** respectively.

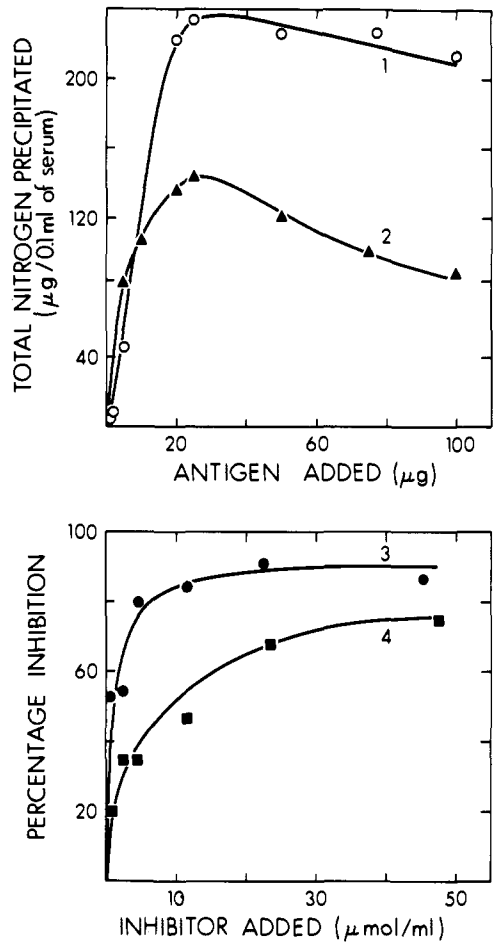


Figure 4. Precipitation and inhibition experiments using the serum of a goat immunized with the blood-group Le^a substance. Plot 1, precipitation by the Le^a glycoprotein. Plot 2, precipitation by the "synthetic" Le^a antigen. Plots 3 and 4, inhibition of the precipitation in plot 1 by increasing amounts of 2-acetamido-2-deoxy-4-*O*-(α-L-fucopyranosyl)-3-*O*-(β-D-galactopyranosyl)-D-glucose and 2-acetamido-2-deoxy-6-*O*-(α-L-fucopyranosyl)-3-*O*-(β)-D-galactopyranosyl-D-glucose, respectively.

deshielding by these oxygen atoms. This deshielding of H-5″ is similar in origin to the specific deshielding influence of halide ions found for anion-acetylated β-D-glucopyranosyl derivatives in solution.[31] The assignment of the rough quartet (since $J_{H\text{-}4'',H\text{-}5''} \sim 0$ Hz) signals to the H-5″ atoms was in each case firmly established by spin decoupling. Consideration of Table II shows that H-5 of the fucosyl group in **20** is 0.47 ppm to lower field than the signal for H-5 in methyl α-L-fucopyranoside, and this result is attributed to arise mainly because of the proximity of 3-O in this disaccharide. The position of H-5 of the fucosyl group in the trisaccharide structure **35** is 0.79 ppm to lower field than H-5 of methyl α-L-fucopyranoside, and this deshielding is attributed to the proximity of O-5′ and O-4′ as well as O-3 to H-5″ (see Figure 2). Thus, an ability to synthesize complex oligosaccharide structures coupled with modern physical methods augurs well for the assessment of the conformational properties of important biological templates in solution.

Having synthesized the saccharide esters **5, 19, 28,** and **36,** it was possible to convert these to the acyl azides, (**8, 22, 31,** and **39,** respectively) using a standard procedure[32] and thereby attach these haptens to a suitable carrier molecule; namely, crystalline bovine serum albumin (BSA). This protein possesses 57 free amino groups per mole (~65,000)[33] and has been widely used for the preparation of so-called "synthetic" antigens. In this work, the immunogen was administered to test animals with Freund's complete adjuvant.[34]

It proved readily possible to raise antibodies to the "synthetic" antigens **9, 23, 32,** and **40** in rabbits. Consideration of the results obtained is restricted in this paper to serum obtained using the trisaccharide antigen **40.** This antigen (degree of substitution on the BSA was 30 haptens per mole) was used to immunize both goats and rabbits. In both cases the antibodies produced showed no precipitation with BSA on immunodiffusion analysis using agarose gel.[35]

As seen from Figure 3, a typical precipitation curve (plot 1) was obtained on adding increasing amounts of the "synthetic" antigen to unit volumes of the rabbit serum. Figure 3 also shows that, as expected, addition of the salt (**37**) of the hapten strongly inhibited the precipitation (plot 2). The salt of the *N*-acetyl-β-D-glucosamine hapten **6** provided no appreciable inhibition. However, both the disaccharide salts **20** and **29** inhibited the precipitation, but the inhibition provided with the β-D-galactosyl group as the terminal unit, as seen in Figure 3 (plot 3), was substantially stronger. It thus appears that the combining site is more concerned with the galactose than with the fucose residue.

The data in Figure 4 are concerned with the establishment of cross-reactions between antibodies raised against the "synthetic" antigen **40** and the antigenic human blood-group Le^a substance.[36]

This glycoprotein was used to immunize a goat,[37] and the development of Le^a specific antibody is demonstrated by the precipitation observed on adding the material to the goat serum (Figure 4, plot 1). Plot 2 of this figure shows that addition of the "synthetic" antigen also caused precipi-

tation and to an extent near 60% of the protein precipitated by the natural glycoprotein. No precipitate was observed on adding either antigen to the goat's serum prior to immunization. Thus, the expected cross-reactions were achieved and provide immunochemical evidence in support of the structures synthesized. Plot 3 of Figure 4 shows the inhibition of precipitation caused by the Le^a trisaccharide whose synthesis was reported in a separate communication in this issue.[6] The positional isomer of this trisaccharide which has the fucosyl group at the 6- rather than the 4-position of the glucosamine residue was also synthesized. Plot 4 of Figure 4 shows that this compound, as would be expected, is a weaker inhibitor. However, the inhibition was quite effective and appears to support the evidence derived from Figure 3 that the galactose residue dominates the Le^a antigenic determinant.

Finally, it may be noted that the serum of a goat immunized with the "synthetic" antigen **40,** after appropriate treatment, effectively agglutinated human blood-group Le (a+ b−) red cells.[38]

Research is continuing in this laboratory toward achieving the synthesis and conformational properties of other antigens related to the human blood groups.

Experimental Section

General. All solvent removals were made using a rotary evaporator and the vacuum of a water pump. Unless otherwise stated, proton magnetic resonance (^1H NMR) spectra were measured at 100 MHz using deuteriochloroform as solvent. The ^{13}C nuclear magnetic resonance spectra (^{13}C NMR) were measured at 22.6 MHz. Thin layer chromatograms (TLC) were made on CAMAG (CH-4132 Muttenz, Switzerland) precoated silica gel DF-B plates and visualized by spraying with 5% sulfuric acid in ethanol followed by heating at 100°. All cation removals employed Rexyn 101 in the H^+ form as supplied by the Fisher Scientific Company, Fair Lawn, N.J. The crystallized bovine serum albumin was supplied by Miles Laboratories Inc., Kankakee, Ill.

8-Ethoxycarbonyloctanoic Acid (1). Concentrated sulfuric acid (21 ml) was added to 98% ethanol (131 ml, 2.25 mol), and the hot solution was added to azelaic acid (282 g, 1.5 mol). The mixture was kept at near 130° until the acid melted to provide two liquid phases, and this mixture was vigorously stirred for 16 hr at 115–120°. The cooled mixture was diluted with diethyl ether (500 ml). The ether layer was washed four times with cold water (500 ml), dried over sodium sulfate, and concentrated to a syrup. Water (100 ml) was added, and sodium carbonate was slowly added with vigorous stirring. The addition was monitored with a pH meter, and the mixture was not allowed to exceed pH 8. The addition was stopped when the pH was stable at pH 7.5. The mixture was extracted three times with diethyl ether (300 ml). The aqueous solution was made acid (near pH 1) with concentrated hydrochloric acid and then extracted three times with diethyl ether (300 ml). The combined extracts were washed three times with water (800 ml) and then dried over sodium sulfate. Solvent removal left a crystalline solid, mp ~25° (lit.[39] mp 27–29°) with ^1H NMR and elemental analysis consistent with the assigned structure. The yield was 107 g (33%).

Anal. Calcd for $C_{11}H_{20}O_4$: C, 61.09; H, 9.32. Found: C, 60.81; H, 9.02.

8-Ethoxycarbonyloctanol (2). Compound 1 (23.6 g, 109 mmol) was dissolved in anhydrous tetrahydrofuran (50 ml) kept in an atmosphere of dry nitrogen. A 1 *M* solution of diborane (prepared according to Brown and coworkers;[40,41] 58 ml) was added dropwise to the stirred solution kept at −15°. The addition was completed in 20 min, and the mixture was maintained at −15° for a further 2 hr prior to leaving overnight at room temperature. Ethanol (20 ml) was added, then 80% aqueous acetic acid (20 ml). The solvents were removed after 30 min, and the residual syrup was suspended in water (100 ml). The pH was brought to 7.5 by the addition of solid sodium carbonate and the mixture then extracted three times with diethyl ether (100 ml). The combined extracts were washed with water (100 ml) and then dried over sodium sulfate. Solvent removal left an oil (19 g, 86% yield) of the title compound of sufficient purity (integrated ^1H NMR) for the preparation of **4,** and no further purification was required.

Anal. Calcd for $C_{11}H_{22}O_3$: C, 65.31; H, 10.93. Found: C, 65.31; H, 10.94.

8-Ethoxycarbonyloctyl 2-Acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-β-D-glucopyranoside (4). Mercuric cyanide (31.8 g, 126 mmol) and anhydrous calcium sulfate (Drierite, 45 g) were added to a solution of **2** (25 g, 124 mmol) in 100 ml of dry benzene. The mixture was protected from moisture while stirring for 1 hr at room temperature prior to addition of 2-acetamido-3,4,6-tri-*O*-acetyl-2-deoxy-α-D-glucopyranosyl chloride[42] (**3**) (25 g, 68 mmol). The mixture was efficiently stirred for 4 days at room temperature. Dichloromethane (400 ml) was added, the solids were removed by filtration, and the filtrate was sequentially washed with 10% aqueous sodium chloride solution (50 ml), once with saturated aqueous sodium bicarbonate solution (25 ml), and twice with water (50 ml). In each case, the aqueous layer was back-extracted with a little dichloromethane. After drying over magnesium sulfate, the solvents were removed to leave a syrup which crystallized from a mixture of diethyl ether and *n*-hexane. The crude yield (28.9 g) was 80%. A recrystallized sample showed mp 112°, $[\alpha]^{20}D$ −12.2° (*c* 2.4, chloroform).

Anal. Calcd for $C_{25}H_{41}NO_{11}$: C, 56.50; H, 7.78; N, 2.63. Found: C, 56.66; H, 7.67; N, 2.83.

The ^1H NMR was consistent with the structure assignment.

8-Ethoxycarbonyloctyl 2-Acetamido-2-deoxy-β-D-glucopyranoside (5). Triethylamine (27 ml) was added to a cooled solution of **4** in 500 ml of methanol and 100 ml of water. The solution was kept at 0–5° for 18 hr prior to solvent removal. The residue was dissolved in 300 ml of methanol, and 200 ml of the acid resin (prewashed with methanol) was added. After stirring for 30 min, the resin was removed by filtration and washed with methanol. The combined filtrates were evaporated to a residue which hardened to a powder on trituration with diethyl ether (50 ml). The powder (20.7 g, 94% yield) was homogeneous on examination by TLC, and the ^1H NMR spectrum was consistent with the structure assigned. The compound (**5**) resisted crystallization and was best characterized as its derivatives **6** and **7** (see Table I).

A suspension of **5** in 10 ml of 85% hydrazine hydrate was agitated ultrasonically for 30 min and then left for 1 day at room temperature. The resulting gel was dissolved in ethanol prior to solvent removal. Toluene was evaporated from the residue to remove traces of hydrazine. The product (compound 7) crystallized from ethanol (404 mg, 83% yield), mp 200–201°. Recrystallization from ethanol gave a sample, mp 200–201°, $[\alpha]^{26}D$ −22.5° (*c* 1, water).

Anal. Calcd for $C_{17}H_{33}N_3O_7$; C, 52.16; H, 8.50; N, 10.72. Found: C, 51.89; H, 8.22; N, 10.43.

A solution of **5** (405 mg, 1 mmol) in 10 ml of 0.1 *N* aqueous sodium hydroxide was kept at room temperature for 3 hr. The solution was freeze-dried to a powder (compound **6**) which provided the ^1H NMR and ^{13}C NMR spectral parameters presented in Tables I and II, respectively.

8-Methoxycarbonyloctyl 2-Acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (11). Sodium (18 mg) was added to dry methanol (40 ml) and, after reaction was complete, the acetate **4** (1.06 g, 2 mmol) was added. The solution was kept at room temperature for 24 hr prior to deionization using the acid resin. Solvent removal left 753 mg of the product **10** which was not characterized but used directly for the preparation of the title compound. The material, dried in vacuo over phosphorus pentoxide was dissolved in *N,N*-dimethylformamide (DMF) (5 ml) which contained α,α-dimethoxytoluene (2 ml) and *p*-toluenesulfonic acid (25 mg).[43,44] After heating at 40° for 1.5 hr, the solution was cooled and triethylamine added to neutralize the acid. After solvent removal, toluene (5 ml) was added and removed by evaporation. This procedure was repeated twice and the residue then triturated with Skellysolve B (10 ml). The solid was collected and dissolved in dichloromethane (25 ml). The solution was washed three times with water (5 ml), dried over magnesium sulfate, and evaporated to a residue which crystallized from ethanol–petroleum ether. The yield was 481 mg (50%), mp 219°. Recrystallization gave the analytical sample, mp 221°, $[\alpha]^{25}D$ −56° (*c* 1.3, DMF).

Anal. Calcd for $C_{25}H_{37}NO_8$: C, 62.61; H, 7.78; N, 2.92. Found: C, 62.47; H, 7.69; N, 3.04.

The ^1H NMR spectrum was consistent with structure **11.**

8-Methoxycarbonyloctyl 2-Acetamido-3-*O*-acetyl-2-deoxy-β-

D-glucopyranoside (13). Compound 11 (0.64 g, 1.33 mmol) was acetylated using 2.4 ml of a 1:1 mixture of acetic anhydride and pyridine at room temperature for 24 hr. The product (12) was isolated in the usual manner and used directly for the preparation of 13.

The crude 12 was dissolved in 50% aqueous acetic acid, and the solution was heated at 100° for 25 min. Solvent removal left a solid which was recrystallized from ethyl acetate–Skellysolve B. The ^{1}H NMR spectrum of the compound (438 mg, 76% yield), mp 122–123°, $[\alpha]^{30}$D −47.3° (*c* 1.1, chloroform), was devoid of signals for aromatic hydrogens and showed singlet signals with correct relative intensities of the methoxy (τ 6.35), acetamido (τ 7.90), and acetoxy (τ 8.07) groups. The signal for the anomeric hydrogen was at τ 5.41 (spacing 8 Hz).

Anal. Calcd for $C_{20}H_{35}NO_9$: C, 55.39; H, 8.14; N, 3.23. Found: C, 55.62; H, 8.05; N, 2.96.

8-Methoxycarbonyloctyl 2-Acetamido-3-*O*-acetyl-6-*O*-benzoyl-2-deoxy-β-D-glucopyranoside (14). Compound 13 (433 mg, 1 mmol) was added to 7 ml of dichloromethane which contained 1.1 mmol of *N*-benzoylimidazole.[45] The solution was kept at the reflux temperature for 18 hr. The product, isolated in the usual manner, was crystallized from ethyl acetate–Skellysolve B. The yield was 476 mg (88%) of compound, mp 105°, $[\alpha]^{32}$D −42.0° (*c* 1, chloroform). The ^{1}H NMR spectrum was consistent with the introduction of a benzoyl group at position 6 of 13 since the ^{1}H NMR spectrum showed the presence of five aromatic protons, and the C-6 methylene protons were shifted downfield to τ 5.27–5.44.

Anal. Calcd for $C_{27}H_{39}NO_{10}$: C, 60.32; H, 7.31; N, 2.61. Found: C, 60.06; H, 7.29; N, 2.74.

2,3,4-Tri-*O*-benzyl-α-L-fucopyranosyl Bromide (16). This compound was prepared as previously reported[46] from 2,3,4-tri-*O*-benzyl-1-*O*-*p*-nitrobenzoyl-β-L-fucopyranose (15) using *N*-*p*-nitrobenzoylimidazole rather than benzoyl chloride which in our hands provided a near 1:1 mixture of the α and β *p*-nitrobenzoates.

A solution of *p*-nitrobenzoyl chloride (7.65 g, 41.2 mmol) in 30 ml dichloromethane was added dropwise to a stirred mixture of imidazole (6.13 g, 90 mmol) in 80 ml of dichloromethane maintained at 0°. After 15 min, the precipitated imidazole hydrochloride was removed by filtration and the filtrate added to a solution of 2,3,4-tri-*O*-benzyl-α-L-fucopyranose (15 g, 34.4 mmol) in acetonitrile (200 ml). This compound was prepared by benzylation of methyl-α-L-fucopyranoside, using benzyl bromide and sodium hydride in DMF according to the general procedure described by Brimacombe,[47] which was found to be far superior than that previously reported.[46] The solution was heated at 65° for 60 hr, and the product was isolated by the addition of diethyl ether (300 ml) and washing in the usual manner with water and aqueous sodium bicarbonate solution prior to solvent removal. The residue crystallized readily from methanol (16.4 g, 85% yield, mp 134°). On recrystallization, the melting point was raised to 135–136°, $[\alpha]^{26}$D +60.9° (*c* 1, chloroform). These constants for 15 differ substantially from those reported by Dejter-Juszynski and Flowers,[46] mp 120–122°, $[\alpha]^{25}$D +29.4°. The ^{1}H NMR spectrum was consistent with the structure assignment. The β configuration was required by the presence of doublet (spacing, 8 Hz) at τ 4.16 for one hydrogen (H-1) which was coupled to another hydrogen (H-2) which produced a quartet signal (spacings 8 and 9 Hz) at τ 5.84.

Anal. Calcd for $C_{34}H_{33}NO_8$: C, 69.97; H, 5.70; N, 2.40. Found: C, 69.85; H, 5.79; N, 2.15.

8-Methoxycarbonyloctyl 2-Acetamido-3-*O*-acetyl-4-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-6-*O*-benzoyl-2-deoxy-β-D-glucopyranoside (17). A mixture of 14 (1.55 g, 2.9 mmol), tetraethylammonium bromide (630 mg, 3 mmol), diisopropylethylamine (450 mg), and DMF (4 ml) in dichloromethane (20 ml) was added to 16 [freshly prepared from 15 (3.38 g, 5.8 mmol)]. The solution was stirred at room temperature for 3 days, after which time TLC examination no longer showed the presence of 14. Dichloromethane (150 ml) was added, and the solution was washed in the usual manner with water and aqueous sodium bicarbonate solution prior to solvent removal. The syrupy product was used directly for the preparation of the crystalline 18.

8-Methoxycarbonyloctyl 2-Acetamido-4-*O*-(2,3,4-tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-β-D-glucopyranoside (18). The crude 17 (3.78 g) was dissolved in 50 ml of dry methanol, and 5 ml of 0.2 *N* sodium methoxide in methanol was added. After 24 hr at room temperature, the solution was deionized using the acid resin. Solvent removal left an oil which was dissolved in dichloromethane (100 ml). The solution was washed with water and dried over magnesium sulfate prior to evaporation to a residue (1.01 g, 85% yield from 14) which crystallized. Purification was by recrystallization from ethyl acetate and Skellysolve B, mp 145–146°, $[\alpha]^{25}$D −33.6° (*c* 1.2, chloroform). The ^{1}H NMR spectrum confirmed the presence of the benzyl, acetamido, aglyconic, and fucosyl groups.

Anal. Calcd for $C_{45}H_{61}NO_{12}$: C, 66.89; H, 7.61; N, 1.73. Found: C, 66.71; H, 7.78; N, 2.02.

8-Methoxycarbonyloctyl 2-Acetamido-2-deoxy-4-*O*-(α-L-fucopyranosyl)-β-D-glucopyranoside (19). Compound 18 (1.4 g) was dissolved in 75 ml ethanol to which was added 1.4 g of 5% palladium on charcoal, and the solution was hydrogenated at 50 psi and 70° for 3 days. After removal of the catalyst by filtration and evaporation of the solvent, the residue was taken up in 25 ml water and washed twice with 5-ml portions of dichloromethane. The dichloromethane extracts were back-washed with a small amount of water and the combined aqueous extracts lyophilized to yield 850 mg (91%) of 19, $[\alpha]^{25}$D −95.5° (*c* 1.0, water).

Anal. Calcd for $C_{24}H_{43}NO_{12}$: C, 53.62; H, 8.06; N, 2.61. Found: C, 53.76; H, 7.95; N, 2.71.

Saponification of 19, as was described for the preparation of 6, provided the sodium salt 20, the ^{13}C NMR and ^{1}H NMR parameters of which are presented in Tables I and II, respectively.

Reaction of 19 with hydrazine, as was described for the preparation of 7, provided the hydrazide 21 in quantitative yield which was used without further purification.

8-Ethoxycarbonyloctyl 2-Acetamido-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (24). Compound 5 (6.3 g, 15.5 mmol) was added to a stirred solution of anhydrous zinc chloride (7.0 g) in freshly distilled benzaldehyde (100 ml) to which was added anhydrous calcium sulfate (Drierite, 14 g). After 20 hr at room temperature, the solids were removed by filtration. The addition of Skellysolve B (500 ml) to the filtrate caused the precipitation of a gummy precipitate which was triturated several times with Skellysolve B. The residue was then triturated with pyridine (7 ml) prior to the addition of dichloromethane (300 ml). The resulting solution was washed twice with water (25 ml), twice with saturated aqueous sodium bicarbonate solution (25 ml), and twice again with water (25 ml). Solvent removal, after drying over sodium sulfate, left a residue which crystallized from ethanol (4.68 g, 61% yield), mp 219–220°. The melting point was unchanged by recrystallization from aqueous methanol, $[\alpha]^{24}$D −55.5° (*c* 1.3, chloroform). The ^{1}H NMR spectrum was consistent with the assigned structure.

Anal. Calcd for $C_{26}H_{39}NO_8$: C, 63.27; H, 7.96; N, 2.84. Found: C, 63.02; H, 7.75; N, 3.14.

8-Ethoxycarbonyloctyl 2-Acetamido-3-*O*-(tetra-*O*-acetyl-β-D-galactopyranosyl)-4,6-*O*-benzylidene-2-deoxy-β-D-glucopyranoside (26). A solution of 24 (12.1 g, 24.5 mmol) and mercuric cyanide (7.35 g, 30 mmol) in 1600 ml of a 1:1 mixture of benzene and nitromethane was distilled at atmospheric pressure to remove 100 ml of solvent. Calcium sulfate (Drierite, 40 g) and tetra-*O*-acetyl-α-D-galactopyranosyl bromide (25, 12.3 g, 29.6 mmol) were added, after cooling, and the temperature was then maintained at 50° for 20 hr. Compound 25 (10 g, 24.4 mmol), mercuric cyanide (6.15 g), and Drierite (10 g) were then added, and the mixture was stirred a further 20 hr at 50°. TLC examination indicated the absence of 24. The solids were removed by filtration and washed with dichloromethane (800 ml). The combined filtrates were twice washed with 30% aqueous potassium iodide solution (50 ml), twice with saturated aqueous sodium bicarbonate solution (50 ml), and then twice with water (50 ml). After drying over sodium sulfate, the solvent was removed to leave a syrup. The syrup was dissolved in a little hot ethanol and diethyl ether then added to near turbidity. The addition of petroleum ether caused the precipitation of a solid which was crystallized from ethyl acetate–Skellysolve B (17.1 g, 85%, mp 108–109°). Recrystallization from ethyl acetate–diethyl ether afforded pure material, mp 110–111°, $[\alpha]^{27}$D −8.2° (*c* 1.4, chloroform). The ^{1}H NMR spectrum was consistent with the assigned structure.

Anal. Calcd for $C_{40}H_{57}NO_{17}$: C, 58.31; H, 6.97; N, 1.70. Found: C, 58.02; H, 7.05; N, 1.87.

8-Ethoxycarbonyloctyl 2-Acetamido-3-*O*-(2,3,4,6-tetra-*O*-acetyl-β-D-galactopyranosyl)-2-deoxy-β-D-glucopyranoside (27). A solution of compound 26 (7.65 g) in 50% aqueous acetic acid (100 ml) was kept at 100° for 25 min. Solvent removal left a residue which crystallized from ethyl acetate–Skellysolve B (5.56 g, 81%,

mp 151°). Recrystallization from ethyl acetate raised the melting point to 153.5°, $[\alpha]^{16}D$ +9.6° (*c* 1.4, chloroform).

Anal. Calcd for $C_{33}H_{53}NO_{17}$: C, 53.87; H, 7.26; N, 1.90. Found: C, 53.78; H, 7.19; N, 1.74.

8-Ethoxycarbonyloctyl 2-Acetamido-2-deoxy-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (28). A solution prepared from **27** (710 mg), triethylamine (483 mg), methanol (5 ml), and water (1 ml) was kept at 0–5° for 20 hr. Most of the product had precipitated. Solvent removal left a solid which crystallized from methanol (450 mg, 82%, mp 207–208°). Recrystallization raised the melting point to 211–212°, $[\alpha]^{17}D$ −22° (*c* 1, water). The ^1H NMR spectrum was consistent with the assigned structure.

Anal. Calcd for $C_{25}H_{45}NO_{13}$: C, 52.90; H, 7.99; N, 2.47. Found: C, 52.90; H, 8.05; N, 2.75.

Saponification of **28**, as was described for the preparation of **6**, provided the sodium salt **29**, the ^{13}C NMR and ^1H NMR parameters of which are presented in Tables I and II, respectively.

An aqueous solution of **29** was deionized by passage through a column of the acid resin. The product, obtained by freeze-drying, crystallized from methanol–diethyl ether, mp 184°, $[\alpha]^{31}D$ −21.9° (*c* 1.1, water). The elemental and ^1H NMR analyses were consistent with expectation for the free acid form of **29**.

Reaction of **28** with hydrazine, as was described for the preparation of **7**, provided a product (**30**) which was purified by recrystallization from methanol, mp 225–226°, $[\alpha]^{27}D$ −20° (*c* 0.75, water), 89% yield.

Anal. Calcd for $C_{23}H_{43}N_3O_{12}$: C, 49.91; H, 7.82; N, 7.59. Found: C, 49.65; H, 7.82; N, 7.29.

8-Ethoxycarbonyloctyl 2-Acetamido-6-*O*-acetyl-3-*O*-(tetra-*O*-acetyl-β-D-galactopyranosyl)-2-deoxy-β-D-glucopyranoside (33). Acetyl chloride (173 mg, 2.2 mmol) in pure chloroform (5 ml) was added dropwise to a stirred solution of imidazole (300 mg, 4.4 mmol) in pure chloroform maintained at 0°. After 5 min, the imidazole hydrochloride was removed by filtration and the filtrate added to compound **27** (1.47 g, 2 mmol). The solution was refluxed for 3 days. Dichloromethane (100 ml) was added, and the solution was washed three times with water (15 ml). After drying over magnesium sulfate, the solvents were removed to leave syrup which was subjected to chromatographic separation using a silicic acid[48] column (28 × 2.5 cm). The column was first developed with 1:1 ethyl acetate–Skellysolve B (700 ml) and then with this mixture containing 5% ethanol. Soon after the development was begun using this latter solvent, the main band appeared which contained a syrup (1.10 g, 71% yield). The product, $[\alpha]^{31}D$ +6.2° (*c* 1, chloroform), appeared pure on TLC examination (R_f 0.44 using 5:5:1 benzene–ethyl acetate–ethanol).

Anal. Calcd for $C_{35}H_{55}NO_{18}$: C, 54.04; H, 7.13; N, 1.80. Found: C, 53.96; H, 7.11; N, 1.94.

The ^1H NMR spectrum showed the signal for the acetamido group at τ 7.86 and those for the five acetoxy groups at τ 7.92–8.03.

8-Ethoxycarbonyloctyl 2-Acetamido-6-*O*-acetyl-3-*O*-(tetra-*O*-acetyl-β-D-galactopyranosyl)-4-*O*-(tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-β-D-glucopyranoside (34). A mixture of **33** (3.0 g, 3.86 mmol), tetraethylammonium bromide (840 mg, 4 mmol), diisopropylethylamine (600 mg, 4.64 mmol), dichloromethane (25 ml), and DMF (5 ml) was added to **16** [freshly prepared from **15** (3.00 g, 5.15 mmol)], and the solution was stirred at room temperature for 1 day. An additional amount of **16** [freshly prepared from **15** (1.50 g, 2.58 mmol)] dissolved in dichloromethane (5 ml) and DMF (1 ml) was then added, and the solution was stirred for an additional 3 days. Dichloromethane (300 ml) was added, and the product was isolated in the usual manner. Purification was effected by chromatography on a silica gel-7[49] column (75 × 3.5 cm). Development with 1:1 ethyl acetate–Skellysolve B (300 ml) was followed by the same solvent but to which 5% ethanol was added. The first band to appear soon after changing solvent provided a TLC homogeneous syrup (4.36 g, 94%), $[\alpha]^{22}D$ −54° (*c* 1.1, chloroform). The ^1H NMR spectrum was consistent with an introduction of the tri-*O*-benzyl-L-fucopyranosyl group.

Anal. Calcd for $C_{62}H_{83}NO_{22}$: C, 62.35; H, 7.01; N, 1.17. Found: C, 62.17; H, 7.11; N, 1.21.

Attempted Preparation of 8-Methoxycarbonyloctyl 2-Acetamido-4-*O*-(tri-*O*-benzyl-α-L-fucopyranosyl)-2-deoxy-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside. Treatment of **34** (850 mg, 0.71 mmol) with 0.01 *N* sodium methoxide in methanol (12 ml) and deionization and isolation of the product as was described for the preparation of **10** provided a syrup (730 mg) which, on examination by ^1H NMR, appeared to still possess one *O*-acetyl group. A second treatment failed to provide *O*-acetyl-free product. The product is provisionally assigned structure **35**.

8-Methoxycarbonyloctyl 2-Acetamido-2-deoxy-4-*O*-(α-L-fucopyranosyl)-3-*O*-(β-D-galactopyranosyl)-β-D-glucopyranoside (36). The crude product **35** was dissolved in ethanol (50 ml), and 5% palladium on charcoal (500 mg) was added. The mixture was shaken in a hydrogen atmosphere at 50 psi and 50° for 2 days. The product, isolated in the usual manner, was dissolved in 0.01 *N* sodium methoxide in methanol solution (10 ml), and the solution was kept at room temperature for 2 days. The solution was deionized prior to solvent removal, and the residue was dissolved in water (20 ml). This aqueous solution was extracted three times with dichloromethane (5 ml) prior to freeze-drying. The colorless powder (388 mg) appeared homogeneous by TLC (R_f 0.28 using 2:2:1 dioxane–ethyl acetate–water), $[\alpha]^{25}D$ −73.5° (*c* 1, water). The ^1H NMR spectrum was consistent with that of the structure assigned. The ^{13}C NMR spectrum is reported and assigned in Table I.

Saponification as described for the preparation of **6** provided the salt **37**, the ^{13}C NMR and ^1H NMR parameters of which are reported in Tables I and II, respectively.

Treatment of **36** with hydrazine and isolation of the product, as was described for the preparation of **7** except that reaction appeared complete (TLC) after 7 hr, gave **38** in quantitative yield as a white powder obtained by freeze-drying of an aqueous solution. TLC examination indicated a pure product (R_f 0.28 using 7:2:1 2-propanol–water–concentrated ammonium hydroxide), $[\alpha]^{25}D$ −72.5° (*c* 1, water).

Preparation of the Antigens 9, 23, 32 and 40. The procedure used was essentially that developed by Inman and coworkers[32] for the attachment of peptide haptens to proteins and involves the use of the hydrazides **7**, **21**, **30**, and **38**, respectively. The hydrazide (0.07 mmol) was dissolved in DMF (1 ml), and the solution was cooled to −25°. A 4 *N* solution of hydrogen chloride in dioxane (0.07 ml, freshly prepared) was added and then *tert*-butyl nitrite (10 mg, 0.1 mmol) in DMF (0.1 ml). After 30 min, sulfamic acid (7 mg), in DMF (0.1 ml) was added, and the stirring was continued for 15 min. The acyl azides (**8**, **22**, **31**, and **39**) were not isolated but the cold solutions used directly in the preparation of the antigens.

Bovine serum albumin (70 mg) was dissolved in an aqueous solution 0.08 *M* in $Na_2B_4O_7$ and 0.35 *M* in $KHCO_3$ and cooled to 0°. The cold solution of the acyl azide was then added dropwise with stirring. The pH of the solution remained between 9.05 and 9.30 during the course of the addition. The solution was dialyzed against at least five changes of deionized water in a Diaflo ultrafiltration cell equipped with a PM-10 membrane[50] and freezed-dried to provide **40** as a white powder.

The hapten-BSA antigens were analyzed for 6-deoxyhexose by the method of Dische and Shettles[51] and for hexose by the phenol–sulfuric acid method[52] correcting for the absorbance due to deoxyhexose. Hexosamine was determined by the modified Morgan–Elson reaction,[53] following acid hydrolysis of the "synthetic" glycoprotein in 4 *M* hydrochloric acid for 4 hr at 100°.

Incorporation of hapten was calculated on a percentage w/w basis, and the number of moles of bound hapten calculated on the basis of a molecular weight for BSA of 65,000 (Table III).[33]

Immunization of Rabbits. Groups of four to six San Juan rabbits[54] were immunized with each carbohydrate BSA conjugate (compounds **9**, **23**, **32**, and **40**) incorporated into Freund's complete adjuvant (FCA). The amount of conjugate administered and the immunization schedule followed the protocol described by Martineau et al.[16]

Immunization of Goats. A female goat was immunized with human blood-group Le^a substance (N-1-2 described by Kabat et al.)[55,56] according to the method of Marcus and Grollman.[37]

A second female goat was immunized with the "synthetic" Le^a antigen (**40**). Antigen (16 mg) in 5 ml of saline emulsified with an equal volume of FCA was injected intramuscularly and subcutaneously at multiple sites on day 1. On day 22, antigen (7.5 mg) incorporated into 5 ml of FCA was administered in a similar fashion. A trial bleeding (day 31) revealed a strong response to the antigen as judged by immunodiffusion in agarose gel.[35] A third injection of antigen (6 mg) (day 42) gave (day 52) immune serum capable of

Table III

Anal.	Compd 9	23	32	40
		Percentage Sugar		
2-Acetamido-2-deoxyglucose	9.4	9.9	8.2	7.8
Fucose		7.5		6.6
Galactose			6.7	7.5
		Degree of Substitution		
	34	38	30	30

precipitating the Le^a glycoprotein (Kabat N-1-2) as well as the immunizing antigen (see Figure 3). After removal of heteroagglutinins,[38,57] both this serum and that from the goat immunized with the Le^a glycoprotein agglutinated (++) human Le (a+ b−) red cells.

Quantitative Precipitin Tests. Quantitative precipitin tests were performed in duplicate according to standard procedures.[58] Increasing amounts of antigen dissolved in phosphate buffered saline 0.15 *M* (PBS) were added to separate 100-μl aliquots of serum. The washed precipitates were dissolved in 0.01 *M* sodium hydroxide aliquots analyzed for protein by the micro Lowry method.[59] Total nitrogen was calculated by reference to a standard of rabbit γ-globulin.[60]

Hapten inhibition experiments were performed in the region of slight antibody excess. Hapten dissolved in PBS was added to 100 μl of serum and incubated at 37° for 30 min. Following the addition of antigen, the solutions were incubated at 37° for 1 hr and then at 4° for 3–5 days. The washed precipitates were analyzed for protein as described for the quantitative precipitin tests.

Acknowledgments. Financial support from the University of Alberta and the National Research Council of Canada (Grant A-172 to R. U. Lemieux) is gratefully acknowledged. Technical assistance was provided by Miss P. Nayar. The ^1H NMR and ^{13}C NMR spectra were measured by the departmental NMR spectroscopy service laboratory under the direction of Dr. T. Nakashima.

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