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Note

Controlled acid hydrolysis of an O-antigen fragment yields univalent heptasaccharide haptens containing one 3,6-dideoxyhexose epitope ‡

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The serology of *Salmonella* has been correlated with defined structural elements located in the bacterial O-antigen[1,2] and the immune response to the most frequently encountered A, B, and D serogroups is often directed toward the 3,6-dideoxyhexose constituent of LPS. A crystal structure of an antibody Fab fragment complexed with a three-repeating unit, dodecasaccharide from the *Salmonella essen* O-antigen revealed the immunodominant monosaccharide, abequose (3,6-dideoxy-α-D-*xylo*-hexopyranose) to be completely buried in a pocket-like binding site[3]. The size of this pocket was sufficiently large to accommodate the galactose and mannose residues of the trisaccharide epitope α-D-Gal-(1 → 2)-[α-D-Abe-(1 → 3)]-α-D-Man, although portions of both were solvent exposed. The polysaccharide antigen which is composed of a branched tetrasaccharide repeating unit,

→ 3)-α-D-Gal*p*-(1 → 2)-[α-D-Abe*p*-(1 → 3)]-α-D-Man*p*-(1 → 4)-α-L-Rha*p*-(1 →

enters and exits the binding site approximately perpendicular to the interface of the two variable domains, V_L and V_H. During the refinement of this crystal structure, scattered, unconnected electron density adjacent to the trisaccharide epitope precluded the positioning of the remaining nine hexose residues of the dodecasaccharide. Motion of the oligosaccharide segments located outside the binding site could be the cause of this disorder. Alternatively the failure to observe the whole oligosaccharide could be attributed to frame shifting of the dodecasaccharide, so that the crystals of the complex would contain Fab bound to each of the abequose epitopes present in the three repeating units. It seemed unlikely that

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all three modes of binding could occur because modeling extended ligands into the site, where the flanking hexose residues adopted low-energy glycosidic torsional angles[4], resulted in severe overlap between the van der Waals surfaces of the antibody and the hexoses at the reducing terminus of the oligosaccharide (Fig. 1A). The residue clashing with the antibody surface was the central galactose, and its position was most strongly influenced by the Man–Rha and Rha–Gal glycosidic torsional angles. Either of these linkages must adopt relatively high-energy values to avoid unfavourable antibody–oligosaccharide contacts or the repeating unit at the reducing terminus must bind (Fig. 1B). Consequently binding to the nonreducing terminus would be strongly disfavoured. In order to test this hypothesis, large univalent ligands representing two repeating units were required.

Heptasaccharides **1** and **2** (2 polymer repeating units minus a single abequose residue) were regarded as candidate univalent haptens. Since the dodecasaccharide and its homologues, octa- up to icosa-saccharide were obtained from the polysaccharide antigen in decreasing yield by the action of a phage associated α-L-rhamnosidase[5], the octasaccharide **3** represented the most abundant material from which to prepare a large univalent hapten. Controlled acid hydrolysis of the acid-labile 3,6-dideoxyhexose residues[6] from the octasaccharide **3** would yield the simplest set of oligosaccharide fragments, and since the two abequose residues should be liberated from the two mannose residues at comparable rates, heptasaccharides **1** and **2** should be produced in nearly equal amounts.

Conditions for mild acid hydrolysis were established by performing the reaction in an NMR tube and observing an ^{1}H NMR spectrum every 15 min (Fig. 2). Each heptasaccharide displayed discrete resonances for the β-anomeric proton of the terminal, reducing rhamnose residue (peak B, Fig. 2) so that optimum yields of heptasaccharides **1** and **2** were reached when these resonances were of approximately equal intensity. These criteria were fulfilled by conducting the hydrolysis in 0.05 M trifluoroacetic acid for 3 h, at 60°C at which point four products, octasaccharide **3**, heptasaccharides **1** and **2**, and hexasaccharide **4**, could be isolated.

An initial separation was done by gel-filtration chromatography to remove low molecular weight compounds and simplify the final separation. Chromatography on a strong anion-exchange resin (Dionex) failed to resolve the two heptasaccharides. Reversed-phase chromatography resolved the hydrolysis mixture, although this powerful tool[7] also separated the isomeric mixtures of oligosaccharides into the α and β anomers of the reducing rhamnose residue. The separation resolved the α anomer of **1** (peak 5, Fig. 3) and the β anomer of **2** (peak 7, Fig. 3), but the β anomer of **1** and the α anomer of **2** eluted with the same retention time (peak 6, Fig. 3). Once the anomeric equilibrium of the mixture had been re-established more heptasaccharides could be isolated for a total yield of 3 mgs each.

The ^{1}H NMR chemical shift data for the α and β anomers of heptasaccharides **1** and **2** are given in Table I. All signals for the rhamnose, abequose, and terminal galactose residues could be assigned with the aid of a conventional COSY spectrum. The central galactose residue, together with the mannose residues, had

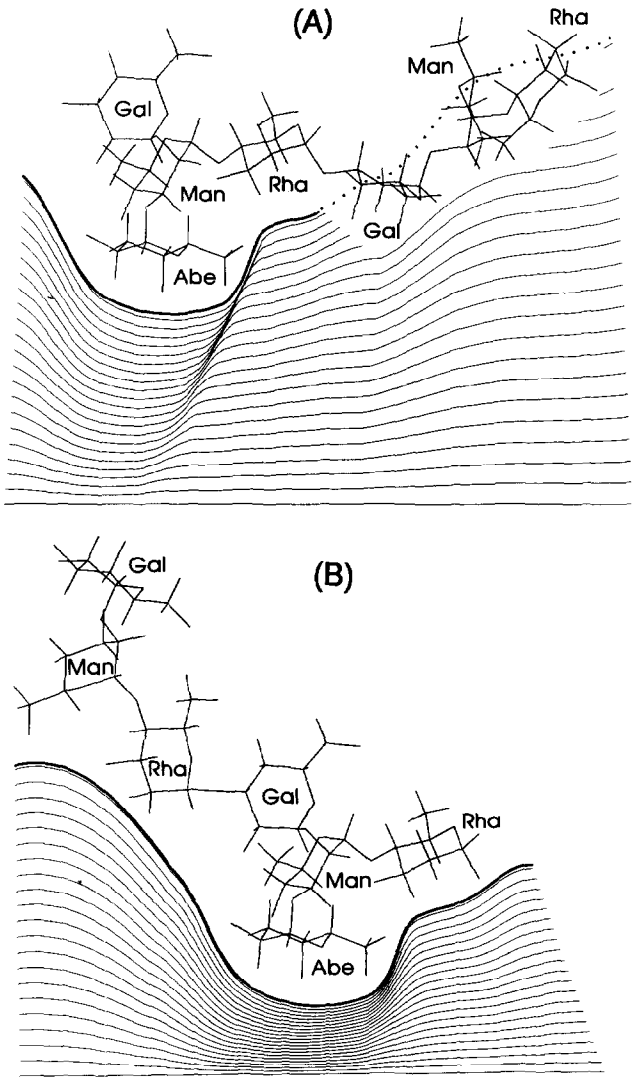
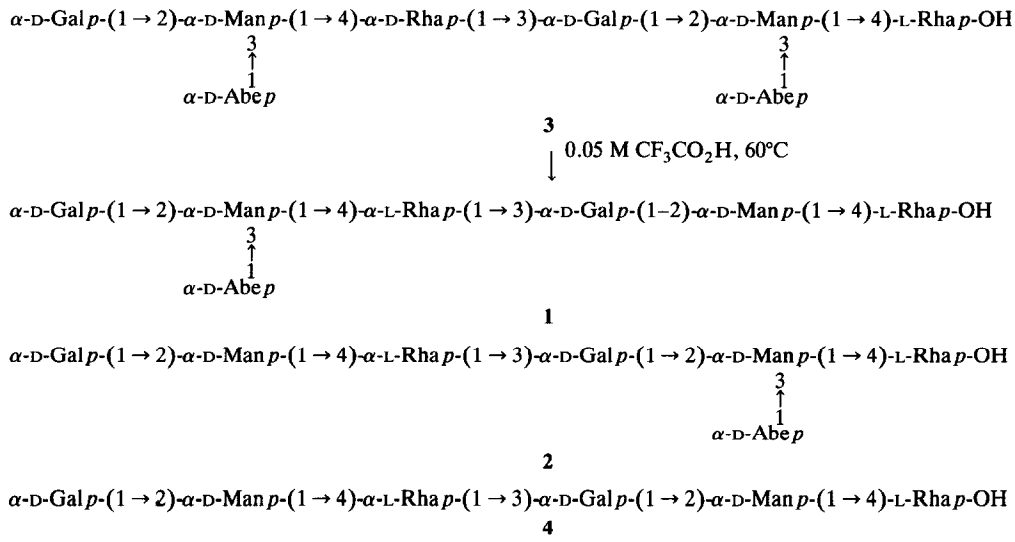


Fig. 1. Two heptasaccharides **1** and **2** positioned with respect to the protein surface of the antibody binding site: (A) Heptasaccharide **1** clashes with the H-2 loop at its reducing terminus if the oligosaccharide adopts the global minimum energy conformation; (B) the nonreducing end of the heptasaccharide **2** shows no unfavourable contacts with the protein in its global minimum-energy conformation.

several of their signals in a very crowded region of the spectrum but could be assigned with the help of a relayed COSY[9], TOCSY[10], and an H–C correlation map (HMQC)[11]. The HMQC spectrum served to identify the C-3 of the mannose residue substituted by abequose, since there is a ~ 8 ppm chemical shift difference between the substituted and unsubstituted carbon signal[4]. The position of the



abequose in each heptasaccharide was confirmed by NOE experiments (ROESY)[12]. A crosspeak between H-1 of the terminal galactose and H-5 of the abequose confirmed the identity of heptasaccharide **1**, and a similar crosspeak between H-1 of the middle galactose and H-5 of abequose in heptasaccharide **2** established its structure.

Upon removal of one abequose unit, the mannose H-4 resonance has the largest change in chemical shift (0.2 ppm upfield), while H-3 which is the proton at the linkage position is shifted 0.1 ppm upfield. All other signals show only small or no changes, which suggests that removal of abequose results in only small conformational changes between the octasaccharide **3** and the two heptasaccharides **1** and **2**. Previously published chemical shift data[8] for the synthetic and phage-derived oligosaccharides have been interpreted in terms of low-energy conformational states that are well approximated by the HSEA force field[4,8].

Preliminary data shows that the IgG binds heptasaccharide **2** with an association constant (K_A) 50-fold higher than that observed for heptasaccharide **1**, and attempts are in progress to co-crystallize **2** with Fab in order to shed light on the role of the flanking hexose residues in determining complex stability.

EXPERIMENTAL

NMR spectra were recorded on a Bruker AMX 600 spectrometer using a broadband probe with the ^{1}H coil closest to the sample. Spectra were recorded at 300 K in 5-mm tubes at concentrations of 3 mg in 0.5 mL D_2O with acetone as the internal chemical shift reference for ^{1}H NMR (δ 2.225 ppm). All experiments were

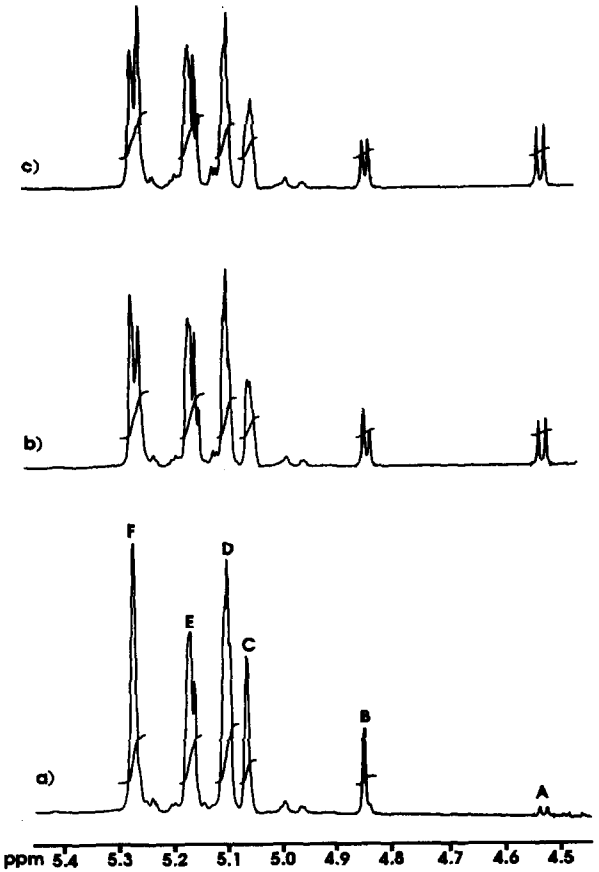


Fig. 2. Anomeric region of 600 MHz ^{1}H NMR spectra illustrating the progress of the acid hydrolysis reaction as octasaccharide **3** is cleaved to yield the heptasaccharides **1** and **2**. Spectrum a: the reaction was followed by observing the changes in the anomeric proton of the β anomer (peak B) of the terminal reducing rhamnose residue (after 5 min). Spectrum b: the signal shifted upfield 0.01 ppm when the abequose close to the reducing end was hydrolyzed (after 90 min). Spectrum c: since both abequoses were hydrolyzed at approximately the same rate, the reaction was 50% complete when both peaks had the same height (after 180 min).

carried out without sample spinning, and standard Bruker software was used. The two-dimensional experiments used for assignment and identification of the compounds were as follows: COSY, Relayed[9], TOCSY[10] ROESY[12], and HMQC[11].

The octasaccharide was obtained from phage-degradation of the *Salmonella* serogroup B polysaccharide as previously reported[5]. Partial hydrolysis was performed by treating the octasaccharide (16 mg) with 0.05 M TFA in D_2O for 3 h at 60°C. The reaction was done in a 5-mm NMR tube in the spectrometer, a spectrum recorded every 15 min, and the reaction was stopped when ~ 50% of the abequose was hydrolyzed, as indicated by the intensity of the H-1 resonances of the reducing rhamnose residues (peak B, Fig. 2) of compounds **1** and **2**, illustrated

TABLE I

^{1}H NMR chemical shift data [a] of heptasaccharides **1** and **2**

Residue		A [b]							B [b]						
		1	2	3	4	5	6	6′	1	2	3	4	5	6	6′
Mannose	**1**	5.318	4.005	4.037	4.017	3.982	3.806	3.864	5.318	4.039	3.944	3.826	3.937	3.806	3.864
	2	5.312	4.024	3.927	3.805	3.946	3.794	3.860	5.319	4.008	4.055	4.028	3.967	3.818	3.867
Galactose	**1**	5.162	3.784	3.916	3.996	4.072	3.703	3.749	5.180	3.954	3.954	4.060	4.112	3.700	3.728
	2	5.158	3.821	3.909	3.990	4.090	3.69	3.75	5.174	3.898	3.946	4.060	4.091	3.69	3.75
Rhamnose	**1**α [c]	5.041	4.075	3.975	3.555	3.932	1.33		5.097	3.920	3.881	3.523	3.957	1.33	
	1β [c]								4.852	3.926	3.708	3.451	3.503	1.32	
	2α [c]	5.038		4.058	3.944	3.550	3.912	1.332		5.107	3.927	3.905	3.521	3.978	1.315
	2β [c]									4.862	3.935	3.734	3.451	3.526	1.330
Abequose	**1**	5.094	4.031	1.973	3.897	4.123	1.175								1.988
	2								5.103	4.033	1.973	3.870	4.114	1.177	
											1.988				

[a] Recorded at 300 K in D_2O with acetone as internal chemical shift reference (δ 2.225 ppm). [b] **A** denotes residues in the repeating unit at the nonreducing end and **B** denotes residues in the repeating unit at the reducing end. [c] The rhamnose residue at the reducing end exists as both the α and β anomer.

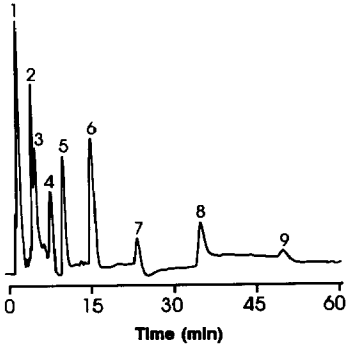


Fig. 3. The HPLC separation of the hydrolysis products including liberated abequose on a C_{18} reversed-phase column using water as the mobile phase. The peaks eluted in the order: 1, abequose α; 2, hexasaccharide **4**α; 3, abequose β; 4, hexasaccharide **4**β; 5, heptasaccharide **1**α; 6, heptasaccharide **1**β + heptasaccharide **2**α; 7, heptasaccharide **2**β; 8, octasaccharide **3**α; and 9, octasaccharide **3**β.

in Fig. 2. The solution was immediately cooled and lyophilized to prevent further hydrolysis.

The crude hydrolysate was applied to a Bio-Gel P-2 column (1.6 × 80 cm) and eluted with water. The fractions which included hexa-, octa-, and the two heptasaccharides were pooled. Separation into the individual compounds was achieved by C_{18} reversed-phase HPLC (Beckman Ultrasphere column ODS 4.6 × 250 mm with 5-μm particle size attached to an LKB instrument, model 2150). The flow rate was 1 mL/min, the mobile phase was deionized water, and 2 mg of the oligosaccharide mixture was separated per run. A Waters refractometer, model R401 modified with capillary tubing, was used for detection. The total yield was 3 mg of each heptasaccharide.

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