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A nuclear magnetic resonance spectroscopic investigation of Kdo-containing oligosaccharides related to the genus-specific epitope of *Chlamydia* lipopolysaccharides

Klaus Bock [a], Jan U. Thomsen [a], Paul Kosma [b], Rudolf Christian [b], Otto Holst [c] and Helmut Brade [c]

[a] *Department of Chemistry, Carlsberg Laboratory, Gl. Carlsberg Vej 10, DK-2500 Valby (Denmark)*
[b] *Institut für Chemie der Universität für Bodenkultur Wien, A-1180 Vienna (Austria)*
[c] *Forschungsinstitut Borstel, Institut für Biologie und Medizin, Parkallee 22, D-2061 Borstel (Germany)*

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ABSTRACT

The ^{1}H- and ^{13}C-NMR parameters, chemical shifts and coupling constants, for the pentasaccharide of the genus-specific epitope of *Chlamydia* lipopolysaccharide and related di-, tri-, and tetra-saccharides have been measured and assigned completely using 1D and 2D techniques, and their structures have been confirmed. NOE experiments indicated the preferred conformation of the pentasaccharide and the component oligosaccharides. The $^{3}J_{H,H}$ demonstrate a change in conformation by rotation of the C-6–C-7 bond of the side chain of the (2 → 8)-linked Kdo (unit *b*) in α-Kdo-(2 → 8)-α-Kdo-(2 → 4)-α-Kdo-(2 → 6)-β-GlcN-(1 → 6)-GlcNol, α-Kdo-(2 → 8)-α-Kdo-(2 → 4)-α-Kdo-(2 → 6)-β-GlcNAc-(1 → O)-allyl, and α-Kdo-(2 → 8)-α-Kdo-(2 → 4)-α-Kdo-(2 → O)-allyl relative to that preferred in α-Kdo-(2 → 4)-α-Kdo-(2 → 6)-β-GlcNAc-(1 → O)-allyl, α-Kdo-(2 → 8)-α-Kdo-(2 → O)-allyl, α-Kdo-(2 → 4)-α-Kdo-(2 → O)-allyl, and α-Kdo-(2 → 6)-β-GlcNAc-(1 → O)-allyl, irrespective of the size of the aglycon, e.g., allyl or β-D-GlcN residues. The conformational results have been substantiated by computer calculations using the HSEA approach.

INTRODUCTION

Chlamydiae are pathogenic, obligatory intracellular parasites which carry on their surface a genus-specific lipopolysaccharide (LPS) antigen that is chemically and antigenically related to the LPS of the Re-mutant of *S. minnesota*[1–3]. Whereas the latter LPS contains, in its carbohydrate moiety, a (2 → 4)-linked disaccharide of 3-deoxy-D-*manno*-octulosonic acid (Kdo)[4], the former contains an additional Kdo residue 8-linked to the terminal Kdo[5]. The gene responsible for the transfer of this third Kdo residue in *C. trachomatis* has been cloned[6] and expressed in the Re-mutant *S. minnesota*[7], resulting in a recombinant LPS which could not be

Correspondence to: Professor K. Bock, Department of Chemistry, Carlsberg Laboratory, Gl. Carlsberg Vej 10, DK-2500 Valby, Denmark.

distinguished serologically from chlamydial LPS. This Kdo trisaccharide, together with the (1 → 6)-linked β-GlcN backbone of lipid A, was isolated from recombinant LPS and identified as the pentasaccharide-alditol α-Kdo-(2 → 8)-α-Kdo-(2 → 4)-α-Kdo-(2 → 6)-β-GlcN-(1 → 6)-GlcNol[8]. Immunochemical studies of this and synthetic partial structures[8,9] showed that the Kdo trisaccharide represents the minimal structure of the genus-specific epitope. Since this epitope is shared by the whole genus *Chlamydia* and not found in any other bacterium, it is important to know how antibodies interact with this structure. The conformational analysis now reported is a prerequisite for this goal.

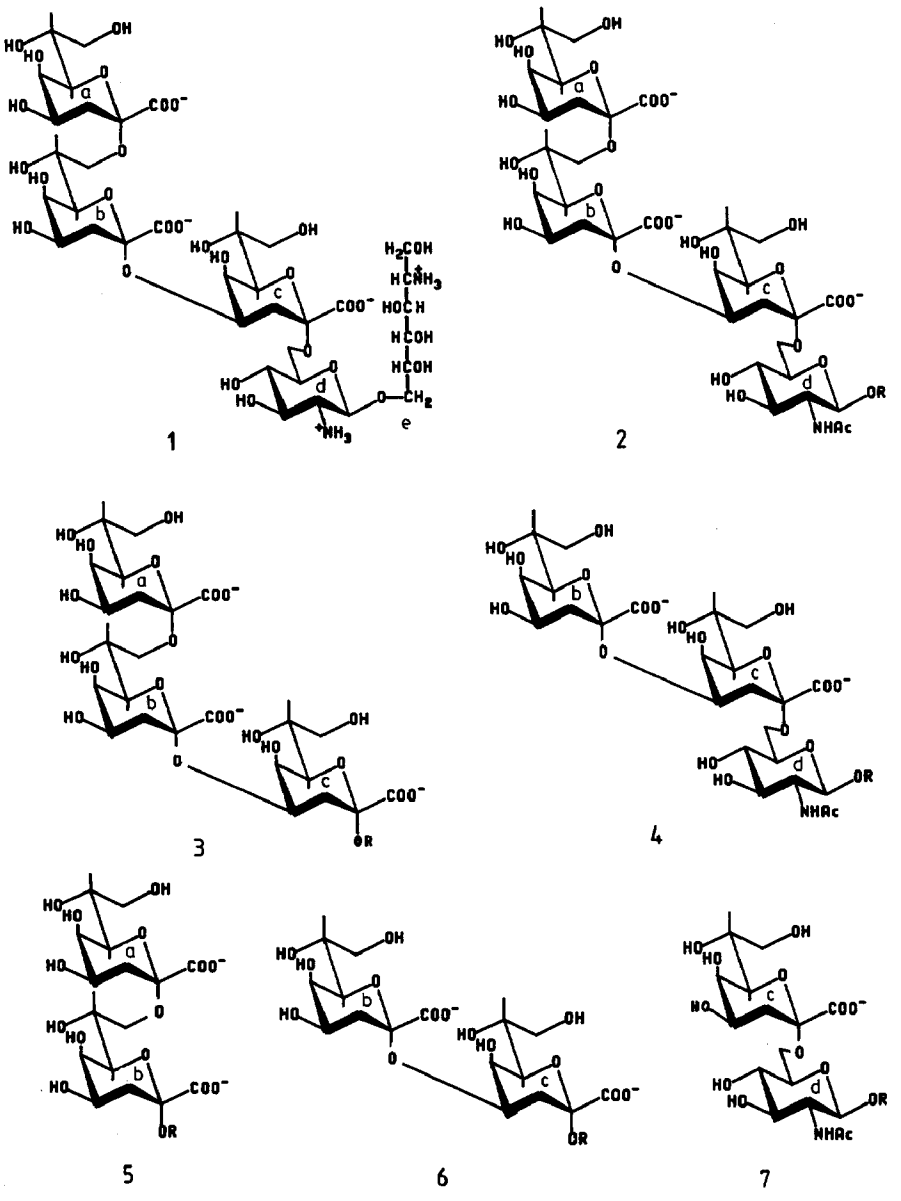
RESULTS AND DISCUSSION

The following compounds were available from previous work[8,10–12]: α-Kdo-(2 → 8)-α-Kdo-(2 → 4)-α-Kdo-(2 → 6)-β-GlcN-(1 → 6)-GlcNol (**1**), α-Kdo-(2 → 8)-α-Kdo-(2 → 4)-α-Kdo-(2 → 6)-β-GlcNAc-(1 → O)-allyl (**2**), α-Kdo-(2 → 8)-α-Kdo-(2 → 4)-α-Kdo-(2 → O)-allyl (**3**), α-Kdo-(2 → 4)-α-Kdo-(2 → 6)-β-GlcNAc-(1 → O)-allyl (**4**), α-Kdo-(2 → 8)-α-Kdo-(2 → O)-allyl, (**5**), α-Kdo-(2 → 4)-α-Kdo-(2 → O)-allyl (**6**), and α-Kdo-(2 → 6)-β-GlcNAc-(1 → O)-allyl (**7**). The identities of **1–7** were confirmed by the NMR data reported in this work.

The ^{1}H-NMR data (500 MHz) for solutions of **1–7** in D_2O at 27° are given in Tables I and II together with those of the reference monosaccharides, allyl α-Kdo (**8**) and methyl β-D-GlcNAc (**9**). The assignments were based on COSY[13], relayed[14], and double-relayed COSY experiments, together with phase-sensitive double-quantum-filtered (DQF) COSY experiments[15]. The ^{13}C-NMR data (125.77 MHz) are given in Table III. The assignments were based on heteronuclear correlation spectroscopy[16] with the assigned proton signals, and by comparison with data for model compounds[17,18]. The 1D NOE data for **3** (Fig. 1A) and **6** (enhancement of 5% of the H-6 resonance of unit *b* on saturation of H-3*eq* of unit *c*) and 2D rotating-frame NOE spectroscopy (ROESY) experiments[19,20] of **1** and **2** (Fig. 1B–D) confirmed the intra-residue interactions and assignments, supported the structures assigned, and confirmed the conformational preference for the (2 → 4) linkage between units *b* and *c*.

Molecular modelling of the preferred conformations of **2–7** were performed using the HSEA approach[21]. The iso-energy contour diagram for the (2 → 4)-linked disaccharide is shown in Fig. 2 to indicate the degree of flexibility possible for this, the most constrained linkage of the oligosaccharide structure, together with the torsional angles for the global minimum (Fig. 3). These results accord with data on the conformational preferences of a rough-mutant lipopolysaccharide from *Escherichia coli*[22]. No attempt was made to calculate the iso-energy contour map for the (2 → 8) linkage due to the expected larger flexibility, conformational averaging, and lack of stereospecific assignment of the protons at C-8.

The *J* values (Table II) indicate that the chair conformations are maintained in the units *a–d* for each compound. However, the orientation of the hydroxymethyl



group of the β-D-GlcNAc unit *d* in the penta- (**1**) and tetra-saccharide (**2**) are different from that in the model monosaccharide[18] with the gt rotamer more populated ($J_{5,6a}$ 8.0 and 6.7 Hz, and $J_{5,6b}$ 2.2 and 2.9 Hz, respectively, cf. 4.8 and 1.6 Hz, respectively, in the model monosaccharide). Furthermore, the orientation of the side chain in the Kdo units, as determined from the values for $J_{6,7}$, $J_{7,8a}$, and

TABLE I

^{1}H-NMR data for Kdo oligosaccharides **1–7**

	1	**2**	**3**	**4**	**5**	**6**	**7**	Reference mono-saccharides [a]
Unit *a*								
α-Kdo-(2 →								**8**
H-3*ax*	1.778	1.780	1.800		1.810			1.800
H-3*eq*	2.028	2.020	2.050		2.050			2.070
H-4	4.080	4.080	4.130		4.060			4.100
H-5	4.062	4.050	4.060		3.990			4.040
H-6	3.673	3.670	3.700		3.590			3.620
H-7	3.962	3.920	3.950		3.930			3.960
H-8a	3.723	3.710	3.720		3.650			3.650
H-8b	3.919	3.910	3.960		3.910			3.940
Unit *b*								
→ 8)-α-Kdo-(2 →								**8**
H-3*ax*	1.798	1.790	1.800	1.740	1.810	1.780		1.800
H-3*eq*	2.120	2.100	2.120	2.130	2.060	2.140		2.070
H-4	4.069	4.060	4.090	4.050	4.110	4.100		4.100
H-5	4.077	4.070	4.080	4.020	4.050	4.050		4.040
H-6	3.789	3.780	3.720	3.590	3.670	3.620		3.620
H-7	4.170	4.160	4.170	3.960	4.000	4.000		3.960
H-8a	3.581	3.580	3.550	3.720	3.630	3.740		3.650
H-8b	3.581	3.580	3.700	3.990	3.630	3.960		3.940
Unit *c*								
→ 4)-α-Kdo-(2 →								**8**
H-3*ax*	1.885	1.880	1.900	1.900		1.920	1.790	1.800
H-3*eq*	2.019	2.020	2.010	2.010		2.020	2.060	2.070
H-4	4.056	4.040	4.070	4.120		4.150	4.070	4.100
H-5	4.103	4.110	4.080	4.090		4.080	4.010	4.040
H-6	3.666	3.690	3.560	3.610		3.560	3.660	3.620
H-7	3.903	3.920	3.920	3.910		3.950	3.940	3.960
H-8a	3.611	3.610	3.620	3.580		3.610	3.630	3.650
H-8b	3.884	3.900	3.920	3.900		3.930	3.920	3.940
Unit *d*								
→ 6)-β-GlcN-(1 →								**9**
H-1	4.419	4.570		4.560			4.530	4.490
H-2	2.631	3.700		3.740			3.700	3.710
H-3	3.364	3.520		3.490			3.490	3.570
H-4	3.329	3.370		3.470			3.430	3.420
H-5	3.592	3.560		3.550			3.560	3.420
H-6a	3.475	3.480		3.570			3.580	3.750
H-6b	3.600	3.600		3.570			3.600	3.900
NAc		2.020		2.020			2.010	2.030
Unit *e*		Allyl glycosides						
→ 6)-GlcN-ol								**8**
H-1a	3.549	4.280	3.920	4.310	3.970	3.930	4.290	3.950
H-1b	3.685	4.130	3.840	4.120	3.870	3.830	4.140	3.850
H-2	3.022	5.920	5.980	5.900	5.980	5.980	5.980	5.970

TABLE I (continued)

	1	2	3	4	5	6	7	Reference mono-saccharides [a]
Unit *e*								
→ 6)-GlcN-ol								
H-3	3.799	5.250	5.210	5.240	5.260	5.230	5.240	5.240
H-3a		5.310	5.320	5.300	5.360	5.350	5.290	5.350
H-4	3.717							
H-5	3.896							
H-6a	3.726							
H-6b	4.112							

[a] **8**, Allyl α-Kdo; and **9**, methyl β-D-GlcNAc.

$J_{7,8b}$ in the model monosaccharide and the oligosaccharides **4–7**, is different from that in unit *b* of **1–3**, where, in particular, the $J_{6,7}$ value of 4.6 ± 0.5 Hz (cf. $\sim 8.5 \pm 0.5$ Hz in the model mono- or' di-saccharides) indicates a substantial change of the rotamer population away from the preponderant *trans* orientation of H-6,7. Furthermore, the values of $J_{7,8a}$ and $J_{7,8b}$ are also changed compared to those for the model oligosaccharides. Inspection of models computed by the HSEA calculations (Fig. 3) suggest that this change in orientation is due most likely to a strong interaction of (2 → 8)-linked Kdo unit *a* and the aglycon of the Kdo unit *c*, which is allyl (**3**), spacer-arm $CH_2CH_2CH_2SCH_2CH_2NHAc$ (data not shown), β-D-GlcNAc (**2**), or β-D-GlcN (**1**). Such a long-range interaction, with the possibility for relief, through multiple minor rotations about bonds in the side chain and the glycosidic linkages, was not expected to result in such a substantial change in conformation. However, the results are supported by data on the binding to monoclonal antibodies in experiments where different component oligosaccharides of the pentasaccharide **1** were used[8,23] and which showed the *Chlamydia*-specific epitope to reside mainly in the Kdo-trisaccharide region. The change of conformation suggested by the *J* values is also reflected in changes in the chemical shifts of the resonances for H-6,7,8a,8b (0.18, 0.21, −0.12 and −0.38 ppm, respectively) of unit *b* in **1–3** when compared to those of the model mono- or di-saccharides.

It was not possible to observe strong inter-unit NOE effects between protons in units *a* and *b* in these compounds, due to the strong mutual relaxation of H-3 and H-8. Partially deuterated derivatives may help to overcome this problem and their syntheses are in progress.

In contrast, the conformational preference for the α-Kdo-(2 → 4)-α-Kdo linkages is much more well defined[22], even though the 4-substitution of a Kdo unit with another Kdo unit causes only minor changes in the chemical shifts of the ^1H resonances. Only the changes in chemical shifts in the ^{13}C-NMR spectra expected for glycosylation are observed. The flexibility of this linkage is shown in Fig. 2 and, in agreement with these results, a strong NOE is observed between H-3*eq* in unit *c* and H-6 in unit *b* in all (2 → 4)-linked Kdo oligosaccharides (see above).

TABLE II

$J_{H,H}$ values [a] (Hz) for Kdo oligosaccharides **1–7**

	1	**2**	**3**	**4**	**5**	**6**	**7**	Reference mono-saccharides [b]
Unit *a*								
α-Kdo-(2 →								**8**
$J_{3ax,3eq}$	−13.0	−13.1	−13.0		−13.0			−13.0
$J_{3ax,4}$	11.4	12.0	12.0		12.0			12.0
$J_{3eq,4}$	5.0	5.0	5.0		5.0			5.0
$J_{4,5}$	2.8		3.0		3.0			3.0
$J_{5,6}$	1.5	1.0	0.8		1.0			0.8
$J_{6,7}$	9.0	8.9	9.5		9.0			9.0
$J_{7,8a}$		6.5	7.0		6.5			7.0
$J_{7,8b}$		2.5	2.5		2.5			3.0
$J_{8a,8b}$	−12.5	−12.0	−12.0		−12.0			−11.5
Unit *b*								
→ 8)-α-Kdo-(2 →								**8**
$J_{3ax,3eq}$	−13.4	−13.1	−13.0	−13.4	−13.0	−13.0		−13.0
$J_{3ax,4}$	13.0	12.0	12.0	11.9	12.0	11.5	12.0	
$J_{3eq,4}$	5.4	5.0	4.5	5.0	5.0	4.5		5.0
$J_{4,5}$	3.0		2.5	3.0	3.0	2.5		3.0
$J_{5,6}$	1.0	0.8	0.8	0.8	1.0	0.5		0.8
$J_{6,7}$	4.0	4.6	5.1	8.9	9.0	8.0		9.0
$J_{7,8a}$	6.8	7.0	8.2	6.8	4.5	7.5		7.0
$J_{7,8b}$	6.8		4.7	2.9	3.5	3.5		3.0
$J_{8a,8b}$			−10.0	−10.8	−10.0	−12.0		−11.5
Unit *c*								
→ 4)-α-Kdo-(2 →								**8**
$J_{3ax,3eq}$	−12.8	−13.1	−12.5	−13.0		−13.0	−13.1	−13.0
$J_{3ax,4}$	11.7	11.7	11.5	11.9		11.5	12.0	12.0
$J_{3eq,4}$	5.2	5.0	4.5	4.9		5.5	5.1	5.0
$J_{4,5}$	2.5	2.9	3.0	2.0		3.0	3.0	3.0
$J_{5,6}$	2.0	1.0	1.0	0.8		0.5	0.5	0.8
$J_{6,7}$	9.2	8.8	8.5	9.0		8.5	9.4	9.0
$J_{7,8a}$	7.2	7.0	7.2			7.0	7.5	7.0
$J_{7,8b}$	3.2	2.5	3.0	2.7		3.2	3.0	3.0
$J_{8a,8b}$	−12.3	−12.0	−12.2	−12.0		−12.0	−12.2	−11.5
Unit *d*								
→ 6)-β-GlcN-(1 →								**9**
$J_{1,2}$	8.2	8.5		8.5			8.5	8.4
$J_{2,3}$	9.5	10.3		10.3			10.1	9.6
$J_{3,4}$	9.1	9.0					8.9	9.2
$J_{4,5}$	9.0	9.7					8.8	9.2
$J_{5,6a}$	8.0	6.7						4.8
$J_{5,6b}$	2.2	2.9						1.6
$J_{6a,6b}$		−10.1						−12.0
Unit *e*								
→ 6)-GlcN-ol								
$J_{1a,1b}$	−11.8							
$J_{1a,2}$	6.3							

TABLE II

$J_{H,H}$ values [a] (Hz) for Kdo oligosaccharides 1–7

	1	2	3	4	5	6	7	Reference mono-saccharides [b]
Unit *e* → 6)-GlcN-ol								
$J_{1b,2}$	4.8							
$J_{2,3}$	6.8							
$J_{3,4}$	2.3							
$J_{4,5}$	8.8							
$J_{5,6a}$	6.1							
$J_{5,6b}$	2.5							
$J_{6a,6b}$	−11.0							

[a] Observed first-order values, accuracy ±0.3 Hz. [b] See footnote to Table I.

The only other significant changes in chemical shifts observed in the tetra- or penta-saccharide are for the α-Kdo-(2 → 6) linkage to the β-D-GlcNAc unit *d*, where the resonances of H-5,6a,6b are shifted by 0.14, −0.3, and −0.27 ppm, respectively. However, in these compounds, a change in the preponderant rotamer of the hydroxymethyl group is observed, as discussed above, and is therefore the most likely reason for these observed differences in chemical shifts.

Thus, the NMR data indicate that there is a conformational transition in the side chain of (2 → 8)-linked α-Kdo oligosaccharides, relative to that in the reference allyl glycoside or in compounds **4–7**, in trisaccharides of the structure α-Kdo-(2 → 8)-α-Kdo-(2 → 4)-α-Kdo-OR where R is an allyl or a monosaccharide unit.

EXPERIMENTAL

Compounds **1–7** were available from previous work[8,10–12].

NMR spectroscopy.—Solutions of ~ 2 mg in 0.5 mL of D_2O at neutral pH were used. Spectra were recorded at 27° in 5-mm tubes at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C with a Bruker AM-500 spectrometer. The ^1H resonances were measured relative to internal acetone (2.225 ppm, DOH at 4.75 ppm) and coupling constants were determined on a first-order basis.

The ^{13}C resonances are relative to internal 1,4-dioxane (67.4 ppm).

Homonuclear 2D NMR spectroscopy was performed with Bruker DISNMRP software. Relayed COSY experiments[14] were made with fixed delays of 30 ms, and double-relayed COSY experiments with both delays of 30 ms in order to optimise coherence transfer for large couplings[24]. These experiments were performed with quadrature detection in the F_1 dimension and a total of 256 t_1 increments of 16 scans each (32 for double-relayed) were recorded with a minimum delay between pulses of 0.1 s and a sweep width of 2500 Hz. The time-domain data matrix was

TABLE III

^{13}C-NMR data for Kdo oligosaccharides **1–7**

	1	**2**	**3**	**4**	**5**	**6**	**7**	Reference mono-saccharides [a]
Unit *a*								
α-Kdo-(2 →								**8**
C-1	175.6	175.7	176.0		176.1			176.1
C-2	100.5	101.4	100.8		101.4			101.1
C-3	35.0	35.0	35.2		34.7			35.1
C-4	66.8	66.8	66.8		66.7			66.9
C-5	67.2	67.2	67.3		67.0			67.2
C-6	72.2	72.1	72.2		72.5			72.5
C-7	70.2	70.2	70.5		70.1			70.4
C-8	63.9	63.9	64.0		63.9			64.1
Unit *b*								
→ 8)-α-Kdo-(2 →								**8**
C-1	176.0	175.9	176.9	176.4	176.7	176.8		176.1
C-2	100.7	100.6	100.8	100.1	100.9	110.2		101.1
C-3	35.3	35.4	35.4	35.3	34.9	35.4		35.1
C-4	66.7	66.7	66.7	66.7	66.8	66.8		66.9
C-5	68.0	68.0	67.9	67.0	67.1	67.2		67.2
C-6	72.4	72.6	73.1	73.3	72.2	73.3		72.5
C-7	71.5	71.3	71.0	70.5	68.5	70.9		70.4
C-8	64.0	64.0	64.6	64.2	65.7	64.0		64.1
Unit *c*								
→ 4)-α-Kdo-(2 →								**8**
C-1	176.4	175.4	176.0	175.4		176.0	175.7	176.1
C-2	101.6	100.7	101.0	100.6		101.0	100.7	101.1
C-3	34.3	34.3	34.2	34.1		34.2	34.9	35.1
C-4	70.9	70.5	70.1	69.2		69.5	66.9	66.9
C-5	65.9	65.6	65.3	65.0		65.2	67.1	67.2
C-6	72.2	72.1	72.3	72.3		72.4	72.4	72.5
C-7	70.4	70.4	70.3	70.4		70.5	70.3	70.4
C-8	64.1	64.1	64.1	64.2		64.0	64.1	64.1
Unit *d*								
→ 6)-β-GlcNAc-(1 →								**9**
C-1	104.5	101.0		101.1			101.0	102.3
C-2	57.5	56.4		56.4			56.3	56.1
C-3	76.8	74.8		75.0			74.8	74.6
C-4	71.4	71.7		71.1			71.3	70.9
C-5	75.2	75.1		75.1			75.0	76.3
C-6	62.9	63.2		62.4			62.8	61.5
NAc		23.0		23.0			23.0	23.0
CO		175.4		175.7			176.0	176.0
Unit *e*		Allyl glycosides						
→ 6)-GlcN-ol								**8**
C-1	63.7	71.4	65.1	71.2	65.1	65.0	71.4	65.4
C-2	54.7	134.3	135.2	134.2	134.6	135.0	134.3	134.8
C-3	70.6	118.6	118.0	118.7	118.3	118.2	118.7	118.8
C-4	71.8							
C-5	70.6							
C-6	72.8							

[a] See footnote to Table I.

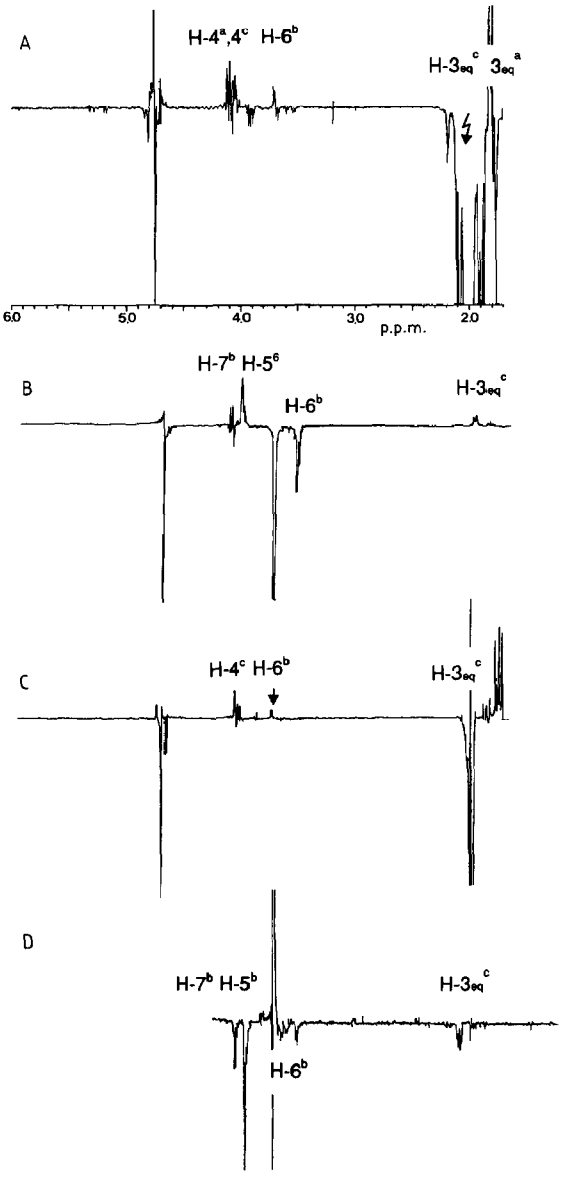


Fig. 1. A, NOE experiment on **3** showing the enhancement of the resonance of H-6 in unit *b* on saturation of H-3*eq* in unit *c*. B–D, Slices of the ROESY spectra of **1** and **2**, which demonstrate the interaction of H-6 and H-3*eq*. No attempts were made to quantitate the enhancements. The superscript letters indicate the residues shown in the formulae.

zero-filled in the t_1 direction to 512×1024 points, treated with a non-shifted sine-bell function in each dimension, and processed to give magnitude spectra.

The phase-sensitive COSY experiments were performed using double-quantum filtering[15,25] with the Bruker COSYPHDQ microprogram and fixed delays of 30 ms. These experiments were performed using 512 t_1 increments and a sweep width

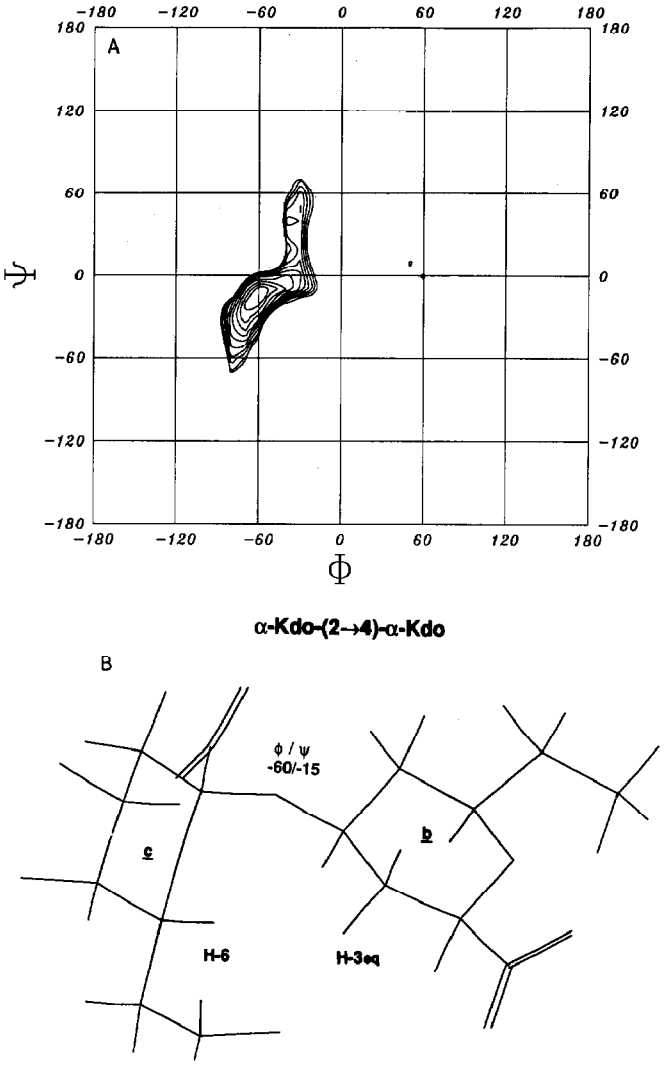


Fig. 2. A, Iso-energy contour diagram (0–10 kcal/mole in 1-kcal steps) for the disaccharide α-Kdo-(2 → 4)-α-Kdo as a function of rotations around ϕ (C-1–C-2–O-2–C-4′) and ψ (C-2–O-2–C-4′–H-4′). The minimum appears at $-60/-15$ ($+/-10$). B, Stick model of the minimum-energy conformation to indicate the short distance between H-3*eq* in unit *b* and H-6 in unit *c* of ~ 2.2 Å.

of 2500 Hz giving an acquisition time in t_1 of 0.205 s. In the F_2 dimension, 2048 data points were collected, giving an acquisition time of 0.819 s. The data matrix was zero-filled in the F_1 dimension to give a matrix of 2048 × 2048 points and was resolution-enhanced in each dimension by a shifted sine-bell function before Fourier transformation.

The $^{13}C-^{1}H$ correlation experiments[16] were performed with the XHCORRD microprogram, using decoupling in the ^{1}H dimension; 128 t_1 increments of 1200 scans and a size of 2048 points were accumulated. The data matrix was zero-filled

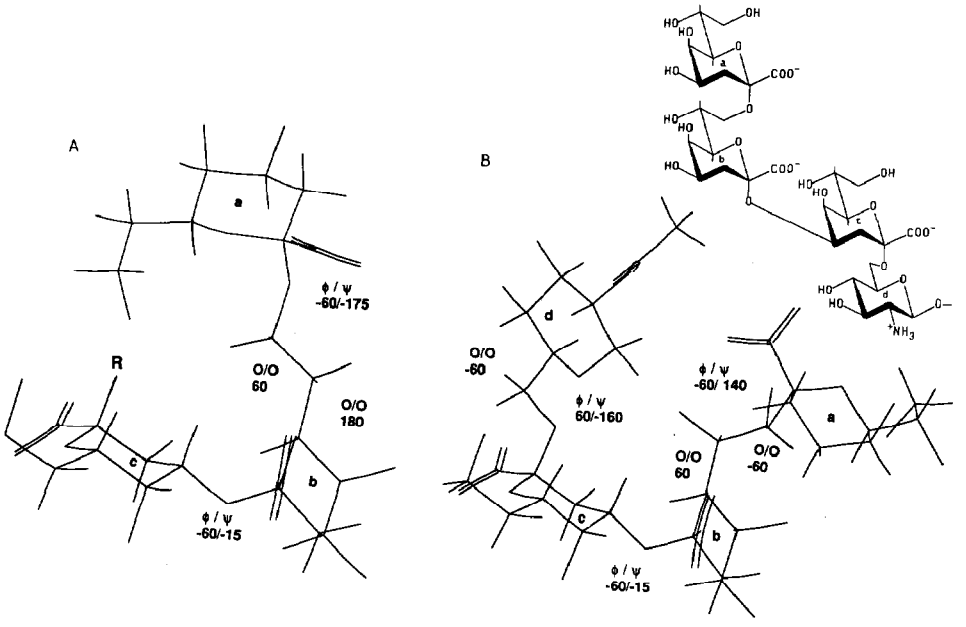


Fig. 3. A, HSEA minimum-energy conformation of **3** with the side chain of unit *b* in the orientation indicated by the X-ray data and in solution, for mono- and di-saccharides of Kdo compounds, indicating the potential interaction of the unit *a* and the aglycon of unit *c*. B, HSEA minimum-energy conformation of **2** with the side chain of the unit *b* in a "*gauche*" orientation as suggested by the observed coupling constants. Minimum energy ϕ,ψ values are indicated on the glycosidic linkages as well as the side-chain orientations (O/O angles).

in the F_1 dimension to 256 × 12048 points before Fourier transform in the absolute mode, giving a digital resolution of 9.8 Hz in the ^{13}C dimension and 11.7 Hz in the ^{1}H dimension.

The ROESY experiments[19,26] were performed as described[27].

HSEA calculations.—These were performed[21] on an IBM PS/2 system model 80 with a 387 math-coprocessor. The torsion angles ϕ and ψ are defined as C-1–C-2–O-2–C-X and C-2–O-2–C-X–H-X, whereas the linkage to the 8(6)-position of the Kdo or glucose unit ψ is C-2–O-2–C-8–C-7 (C-2–O-2–C-6–C-5) and ω is O-6–C-6–C-5–O-5. The co-ordinates for the α-Kdo or β-D-GlcNAc units were taken from the X-ray structures, respectively[28,29], and the protons attached as described[30].

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