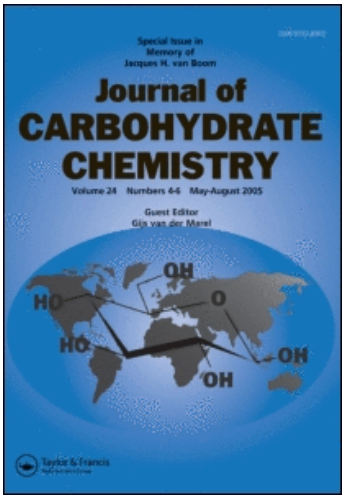

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[a] Istituto di Chimica delle Macromolecole del, Milano, Italy [b] Istituto di Chimica e Biochimica, Milano, Italy [c] Departamento de Bioquimica, Sao Paulo, Brazil

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CONFORMATION OF THE UNSATURATED URONIC ACID RESIDUES OF GLYCOSAMINOGLYCAN DISACCHARIDES [1]

M. Ragazzi,*° D. R. Ferro,° A. Provasoli,° P. Pumilia,° A. Cassinari,°
G. Torri,+ M. Guerrini,+ B. Casu,+ H. B. Nader,§ and C. P. Dietrich§

° Istituto di Chimica delle Macromolecole del CNR
Via E. Bassini 15, I-20133 Milano (Italy)
+ Istituto di Chimica e Biochimica "G. Ronzoni"
Via G. Colombo 81, I-20133 Milano (Italy)
§ Departamento de Bioquimica, Escola Paulista de Medicina
Rua Botucatu 740, 04034, Sao Paulo (Brazil)

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ABSTRACT

Molecular mechanics calculations (using the REFINE package) have been performed on a series of disaccharides obtained by cleavage of glycosaminoglycans with lyases, in order to examine the effect of chemical environment on the conformation of the 4,5-unsaturated uronic acid residue. The disaccharides were derived from heparin and heparan sulfate (**1-5**), hyaluronic acid (**6**), chondroitin (**7**), chondroitin-4-sulfate (**8**), and chondroitin-6-sulfate (**9**). The ^1H NMR spectra were analysed for the above compounds and for the unsaturated uronic acid residues of a low-molecular weight *E. Coli* K5 polysaccharide (**10**), as well as for the alditol derivative (**11**) of compound **8**; the wide variation of the interproton vicinal coupling constants as a function of configuration and position of the glycosidic linkages and of the sulfation pattern is unequivocally interpreted in terms of equilibrium between two distinct ring forms, namely $^2H_1$ and $^1H_2$. The equilibrium is semi-quantitatively explained by the results of the present energy calculations.

INTRODUCTION

The existence of different sugar ring forms has been recognized for a long time, but much remains to be done in order to understand the nature of the forces determining the equilibria among such forms and the dependence of the conformational behavior of sugar compounds on the environment (chemical substitutions, solvent effects). From the point of view of conformational analysis, as carried out by means of molecular mechanics methods, severe problems are still posed by the presence of charged groups (such as $-COO^-$, $-SO_3^-$ and $-NH_3^+$) and more in general of highly polar moieties, giving rise to strong electrostatic interactions. Also less energetic terms, such as the well-known anomeric effect, as well as certain torsional barriers, will require further refinements in order to achieve satisfactory calibration for a sugar-oriented force field.

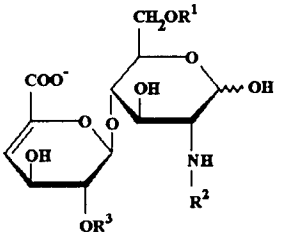
Another level of complexity is added to the problem when sugar rings may adopt more than one conformation with equal energy, as in the case of the iduronate residues in glycosaminoglycan sequences[2] and of the glucuronate ring in chondrosine.[3] Combined NMR and molecular mechanics studies indicated that the conformation of these residues in solution is represented by an equilibrium among different conformers.[2-6]

Reported values of interproton coupling constants for 4,5-unsaturated uronic acids either as monosaccharides [7-12] or as terminal, non-reducing residues of di- and oligosaccharides,[7,10-13] suggest that also these rings might be conformationally flexible. Indeed, the crystallographic evidence that the unsaturated uronic acid residues of the disaccharide 4-*O*-(4-deoxy-β-L-*threo*-hex-4-enosyl)uronate-α-D-galacturonate exist in two different forms ($^2H_1$ and $^1H_2$) in the same unit cell, indicated that these forms are of almost equal energy.[15] A preliminary ^1H NMR study of 4,5-unsaturated disaccharide products of glycosaminoglycan cleavage with lyases (heparinases, heparitinases and chondroitinases) suggested that their unsaturated uronic acid residues are in equilibrium between these ring forms.[16]

Here we present results of a combined ^1H NMR and molecular mechanics study on these disaccharides (**1-9**, shown in Scheme 1[17]), confirming that the conformation of their unsaturated uronic acid residues can indeed be represented in terms of an equilibrium between the $^2H_1$ and $^1H_2$ forms, the relative population of conformers depending on configuration and position of the glycosidic linkages and on the sulfation pattern.

EXPERIMENTAL

Preparation. Disaccharides **1-9** were isolated, as previously described,[12,18,19] by preparative paper chromatography from digests with lyases of heparin, heparan sulfate,



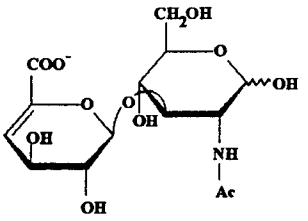
1 $R^1 = R^2 = SO_3^-$; $R^3 = H$

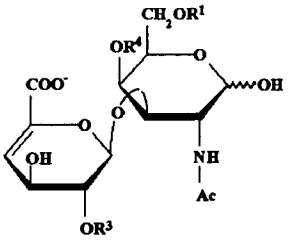
2 $R^1 = R^3 = H$; $R^2 = SO_3^-$

3 $R^1 = SO_3^-$; $R^2 = Ac$; $R^3 = H$

4 $R^1 = R^3 = H$: $R^2 = Ac$

5 $R^1 = R^2 = R^3 = SO_3^-$



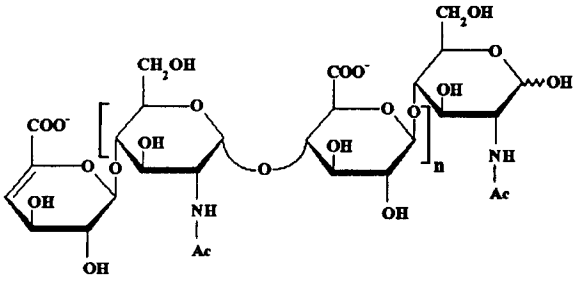


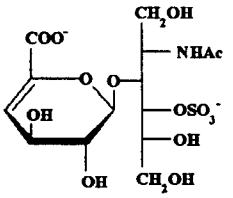
7 $R^1 = R^3 = R^4 = H$

8 $R^1 = R^3 = H$; $R^4 = SO_3^-$

9 $R^1 = SO_3^-$; $R^3 = R^4 = H$

6





10 11

SCHEME 1

hyaluronic acid, chondroitin, chondroitin-4-sulfate, and chondroitin-6-sulfate. The low-molecular weight K5 polysaccharide (**10**)[20] was a gift from Prof. K. Jann (Freiburg). The alditol derivative (**11**) was obtained by borohydride reduction of disaccharide **8**.[21]

NMR spectroscopy. NMR spectra were recorded from solutions in D_2O, at 30° C and 500 MHz for ^1H, and at 125.76 MHz for ^{13}C, with a Bruker AMX-500 spectrometer. Interproton coupling constants were measured from spectra obtained at 23° C and 300 MHz, with the Bruker CPX-300 spectrometer. All proton shifts are referenced to internal

4,4-dimethyl 4-silapentane sodium sulfonate (DSS) and ^{13}C shifts are relative to internal methanol in D_2O (51.68 ppm with respect to external TSP). 2-D homonuclear correlation spectroscopy (COSY-RCT-TOCSY) was used to assign proton spectra, while ^{13}C assignment was performed using ^{1}H detected heteronuclear multiple quantum coherence via direct coupling (HMQC). This approach alleviates the ^{13}C sensitivity problem by at least an order of magnitude.[22]

Method of calculation. All energy calculations were performed with package REFINE, a molecular mechanics program developed in our laboratory.[23] Allinger's force-field MM2/87 [24] was utilized whenever possible; the parameters for the ionic groups $-COO^-$, $-O-SO_3^-$, and $-NH-SO_3^-$ and for the hydrogen bonds were taken from our previous work on heparin oligosaccharides (see Ref. 25 and references therein).

Preliminary calculations on the unsaturated residue led, as expected, to two energy minima, namely the ring forms $^{1}H_2$ and $^{2}H_1$ (Fig.1); their geometrical features are summarized as follows:

	$^{1}H_2$	$^{2}H_1$
Dihedral angle		
C1-C2-C3-C4	44.7	-50.5
C2-C3-C4-C5	-17.4	20.9
C3-C4-C5-O	-1.5	1.4
C4-C5-O-C1	-10.7	9.8
C5-O-C1-C2	41.0	-42.6
O-C1-C2-C3	-59.1	64.1
Puckering angle φ_2	51.5	128.4
„ „ θ	40.5	223.8
„ amplitude Q	0.46	0.52

Exploration of the energy surface $E(\varphi_2,\theta)$, where φ_2 and θ are Cremer and Pople's spherical coordinates,[27,28] yielded no other minimum energy ring conformation. Thus, the $^{1}H_2$ and $^{2}H_1$ forms of the unsaturated residue and the $^{4}C_1$ form of the reducing residue were taken as starting points of all subsequent calculations.

For each compound **1-9** a preliminary search of minimum energy structures as a function of (φ,ψ) was done, for the two ring forms and a reasonable arrangement of the side chains. Then all possible starting structures were built by rotating the side chains with suitable steps for each (φ,ψ) pair, and the energy of each structure was minimized

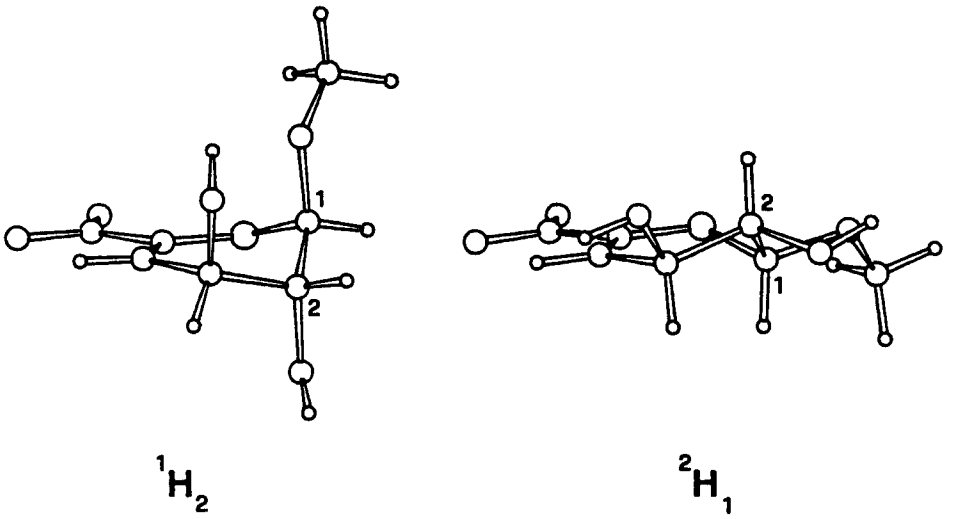


FIG. 1 Representation[26] of the two half-chair forms computed for the 4,5 unsaturated methyl pyranoside. Note the *all-axial* conformation of the substituents in positions 1, 2, and 3 in the $^1H_2$ form, as opposed to the almost flat structure for the $^2H_1$.

with respect to the cartesian coordinates of all atoms for three different values of the dielectric constant (namely, ε = 1, 1.5, and 3). Finally, Boltzmann statistics on the energies were carried out, which produced the theoretical populations of the two forms $^1H_2$ and $^2H_1$.

RESULTS AND DISCUSSION

The ^1H and ^{13}C NMR chemical shifts for the unsaturated uronic acid and the amino-sugar residues of compounds **1-11** are reported in Table 1 and 2, respectively. For reducing disaccharides (**1-9**), data for both the α (upper line) and β anomers are given. The chemical shift data and assignment of the α-anomer of disaccharide **8** and for its alditol derivative **11** agree with those reported in the literature (21).

The NMR spectra are compatible with the structures shown in Scheme 1. The observed vicinal interproton coupling constants for the prevalent (α) anomer of compounds **1-9**, and for the only form of **10** and **11** are listed in Table 3.

In Figure 2, the $J_{2,3}$ and $J_{3,4}$ values are plotted versus $J_{1,2}$. Inspection of the plot shows linear relationships among the J's:

$$J_{2,3} = a + b \cdot J_{1,2} \quad \text{and} \quad J_{3,4} = c + d \cdot J_{1,2},$$

where $a = -0.44 \pm 0.36$, $b = 0.98 \pm 0.07$, $c = 5.67 \pm 0.22$, $d = -0.35 \pm 0.05$ Hz.

Table 1. ^{1}H NMR chemical shifts

Compd	U1	U2	U3	U4	A1	A2	A3	A4	A5	A6 a	A6 b
1	5.169	3.80	4.16	5.829	5.414	3.237	3.69	3.77	4.10	4.30	4.17
	5.169	3.80	4.16	5.829	4.695	3.011	3.68	3.77	4.10	4.30	4.17
2	5.154	3.81	4.236	5.866	5.450	3.258	3.76	3.76	3.75-3.94	3.9	3.84
	5.154	3.81	4.236	5.866	4.711	3.031	3.75	3.57	3.75-3.94	3.9	3.84
3	5.173	3.830	4.183	5.820	5.201	3.889	3.88	3.77	4.13	4.32	4.23
					4.706	3.684	3.76	n.d.	n.d.	n.d.	n.d.
4	5.154	3.813	4.230	5.858	5.206	3.880	3.95	3.76	3.68 (*)	3.9	3.8
	5.154	3.807	4.230	5.852	4.700	3.690	3.85	3.76	3.59 (*)	3.9	3.8
5	5.496	4.559	4.315	5.948	5.435	3.259	3.751	3.80	4.144	4.314	4.21
	5.490	4.560	4.310	5.950	4.694	3.050	3.728	3.80	4.264	4.2 - 4.3	
6	5.182	3.741	4.140	5.863	5.145	4.02	3.87	3.99	3.87	3.73 - 3.8	
	5.164	3.741	4.128	5.857	4.766	3.80	3.49	3.80	3.51	3.73 - 3.8	
7	5.23	3.80	4.09	5.89	5.210	4.27	4.08	4.17	4.12	3.70 - 3.75	
	5.19	3.80	4.09	5.89	4.700	3.98	3.91	4.08	4.13	3.70 - 3.75	
8	5.293	3.84	3.939	5.962	5.202	4.36	4.30	4.68	4.27	3.77	3.69
	5.257	3.82	3.939	5.958	4.742	4.04	4.16	4.61	4.23	3.81	3.74
9	5.23	3.79	4.100	5.81	5.21	4.29	4.13	3.95	n.d.	4.18	4.11
					4.73	3.96	3.94	n.d.	n.d.	4.18	4.11
10	5.101	3.801	4.276	5.810	5.34	3.87	3.84	3.62	3.80	3.82	3.82
11	5.263	3.932	4.160	5.94	a 3.722 b 3.666	4.314	4.365	4.438	4.065	3.677	3.677

(*) assignments interchangeable

Table 2. ^{13}C NMR chemical shifts

Compd	U1	U2	U3	U4	U5	A1	A2	A3	A4	A5	A6
1	103.1	72.3	68.6	110.0	n.d.	93.5	59.9	71.6 (*)	81.2	70.3	68.7
	103.1	72.3	68.6	110.0	n.d.	98.3	63.1	74.7 (*)	78.3	n.d.	68.7
2	102.8	72.7	69.3	110.7	146.8	93.6	60.4	71.8	80.9	72.8	62.7
	102.8	72.7	69.3	110.7	146.8	96.1	63.0	71.8	n.d.	72.8	62.7
3	103.4	72.3	68.3	110.0	n.d.	93.0	57.6	71.5	81.8	70.3	68.7
						97.7	58.2	74.5	n.d.	n.d.	68.7
4	102.8	72.5	69.0	110.2	146.5	92.7	56.2	72.7 (*)	81.2	77.3 (*)	62.3
	102.6	72.5	69.0	110.2	146.5	97.2	58.8	71.4 (*)	80.7	74.6 (*)	62.3
5	99.6	77.0	65.0	109.3	n.d.	93.7	59.3	70.9	81.0	70.5	68.6
	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
6	103.4	72.7	69.0	110.3	147.1	93.7	55.9	74.2	82.6	71.1	68.8
	103.3	72.6	68.8	110.3	147.1	97.2	58.7	78.4	84.8	n.d	68.9
7	104.6	73.4	69.3	110.9	n.d.	94.9	52.8	80.7	72.0	74.0 (*)	65.1
					n.d.	98.6	56.1	83.4	74.0	71.4 (*)	65.1
8	102.6	70.8	68.7	109.3	n.d.	93.8	52.0	76.2	79.5	73.9	63.4
	102.6	70.8	68.7	109.3	n.d.	98.1	55.7	77.8	78.9	72.3	63.4
9	103.7	71.7	67.8	110.0	n.d.	93.6	50.5	78.8	74.8	n.d.	69.8
						97.7	53.6	78.0	n.d.	n.d.	n.d.
10	102.8	77.2/78.8	68.8	109.6	146.3	98.5	54.9	72.4	80.0	70.8	61.1
11	102.9	71.4	67.9	109.3	146.4	62.4	53.6	77.7	80.9	72.4	65.0

(*) assignments interchangeable.

Table 3. Proton coupling constants evaluated from the ^1H NMR spectra.

Compd	Abbreviation	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$
1	ΔUA-GlcNS6S	5.52	4.69	3.90
2	ΔUA-GlcNS	6.05	5.45	3.54
3	ΔUA-GlcNAc6S	5.5	------	3.8
4	ΔUA-GlcNAc (HS) [a]	5.95	5.38	3.48
5	ΔUA2S-GlcNS6S [b]	3.2	2.8	4.4
6	ΔUA-GlcNAc (HA) [c]	4.80	4.55	3.75
7	ΔUA-GalNAc	4.0	------	4.3
8	ΔUA-GalNAc4S	3.11	2.35	4.72
9	ΔUA-GalNAc6S	4.53	4.08	4.12
10	ΔUA-GlcNAc-K5 [d]	6.64	6.00	3.48
11	ΔUA-GalNAc.ol4S	4.6	4.0	4.3

a. from heparan sulfate; b. data from (13); c. from hyaluronate; d. non-reducing terminal disaccharide of LMW K5 (*E. coli*)

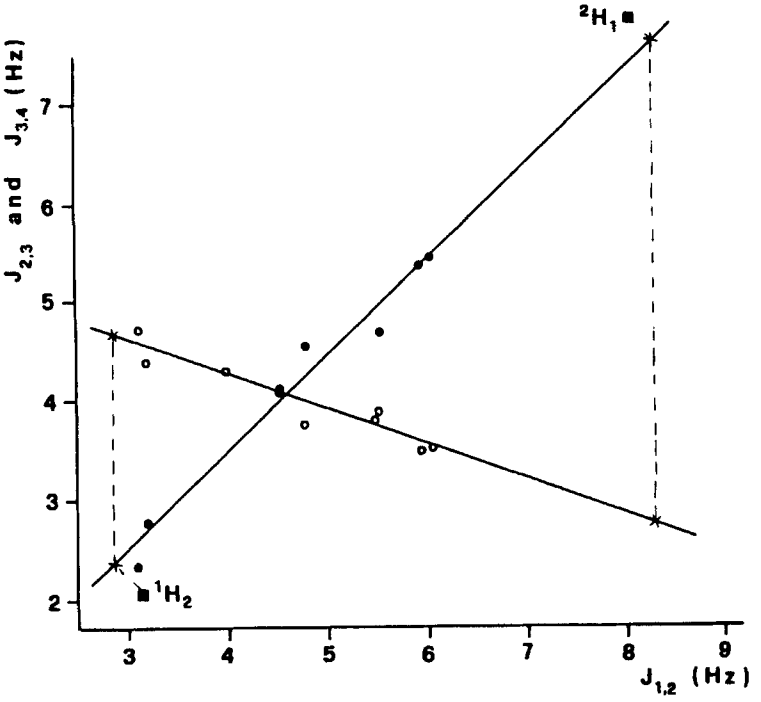


FIG. 2 Plot of observed vicinal coupling constants $J_{2,3}$ (•) and $J_{3,4}$ (o) versus $J_{2,3}$. Theoretical values (■) and interpolated J'(×) for $^1H_2$ and $^2H_1$ forms are shown.

Table 4.

	$^1H_2$			$^2H_1$		
	1,2	2,3	3,4	1,2	2,3	3,4
H-C-C-H	57.9	-74.5	45.0	-175.1	-164.5	73.0
J	3.15	2.09	-	8.08	7.86	-
J'	2.87	2.37	4.67	8.27	7.67	2.78

The above relationships confirm that the coupling constants of the various compounds may be interpreted in terms of equilibrium between only two conformers. Indeed, by assuming the $^1H_2$ and $^2H_1$ forms as the conformers in equilibrium, and applying the Haasnoot *et al.* equation[29] to the dihedral angles H-C-C-H corresponding to the two minimum energy conformers of the unsaturated residue, the theoretical values of $J_{1,2}$ and $J_{2,3}$ are found in good agreement with the straight line best fitting the observed values (Fig. 2).

On the other hand, the energy calculations rule out the possibility that the observed changes in the J's may arise from distortions of a unique conformer. Two distinct energy minima were found for all the investigated compounds, with small deviations (only a few degrees) in the H-C-C-H dihedral angles. The average values of the dihedral angles utilized for computing the vicinal J's are listed in Table 4, together with the calculated coupling constants of the two pure forms.

The conformer populations were first computed by least-square fitting the observed J's to the theoretical values; unfortunately the Haasnoot *et al.* equation has not been parametrized for the unsaturated - CH= moiety, so the $J_{3,4}$ data could not be used in the fitting procedure. In order also to use such data, in a second calculation the theoretical coupling constants were replaced by the closest points lying on the experimental lines (the J' values listed at the bottom of Table 4): this new least-square fitting yields populations very close to the previous one.

Due to lack of a theoretical relationship for $J_{3,4}$, the J' values of 2.78 Hz for $^2H_1$ and 4.67 Hz for $^1H_2$ can confidently be proposed for use in future calculations. The J's calculated with this second method for compounds **1-9**, their rms deviations with respect to the observed values, and the corresponding conformer populations are given in Table 5.

The results show that the equilibrium ranges from the substantially pure form $^1H_2$ (compound **8**) to *ca.* 60% of form $^2H_1$ for compounds **2** and **4**. As for the long-range $J_{1,3}$ and $J_{2,4}$ couplings, significant values have been observed for compounds **8** (1.2 and 1.5 Hz, respectively), **5** (1.1 Hz for both, see also ref. 12), **9** (0.8 Hz for $J_{2,4}$)[30], and **1**

Table 5. Best-fitting coupling constants and percent populations of the $^2H_1$ form of the unsaturated ring, and populations calculated by molecular mechanics.

Compd	$J_{1,2}$	$J_{2,3}$	$J_{3,4}$	J rms	exptl % $^2H_1$	theoretical % $^2H_1$		
						ε=1.	ε=1.5	ε=3.
1	5.36	4.81	3.79	0.13	46.1	95	65	23
2	6.03	5.47	3.56	0.02	58.6	93	60	44
3	5.48	(4.93)	3.75	0.04	48.4	55	29	20
4*	5.96	5.40	3.58	0.06	57.3	45	52	43
5	3.28	2.77	4.52	0.09	7.5	6	17	40
6*	4.98	4.43	3.93	0.16	39.0	23	37	27
7*	3.99	(3.47)	4.27	0.02	20.7	12	14	14
8	2.97	2.46	4.63	0.12	1.8	3	1	1
9	4.56	4.03	4.07	0.04	31.3	27	24	12
10	6.59	6.01	3.36	0.07	68.8	--	--	--
11	4.53	4.00	4.08	0.13	30.7	--	--	--

* Non-sulfated compound

(0.85 and 0.35, respectively): these data, though not exhaustive, nicely agree with the experimental populations of the $^1H_2$ form. The last two rows of Table 5 show data concerning compounds not considered in the present molecular mechanics calculations; also these data, not included in the best-fitting procedure, agree very well with the curves of Fig. 2. The result for compound **10** indicates a shift of 10% towards $^2H_1$ when disaccharide **4** is attached to the K5 (*E. coli*) polysaccharide chain.

Although calculations were performed only on the major anomeric form (α) of disaccharides **1-9**, the experimental coupling constant values for the β anomeric forms (data not reported here) are very close (± .1 Hz), suggesting only minor influence of the configuration on the conformational equilibrium. The conformer populations derived by molecular mechanics are presented in the last columns of Table 5 for three different values of the effective dielectric constant.

The energy calculations confirm the existence of an equilibrium between the two half chair forms $^1H_2$ and $^2H_1$ of the unsaturated ring, and reproduce the trend shown by the

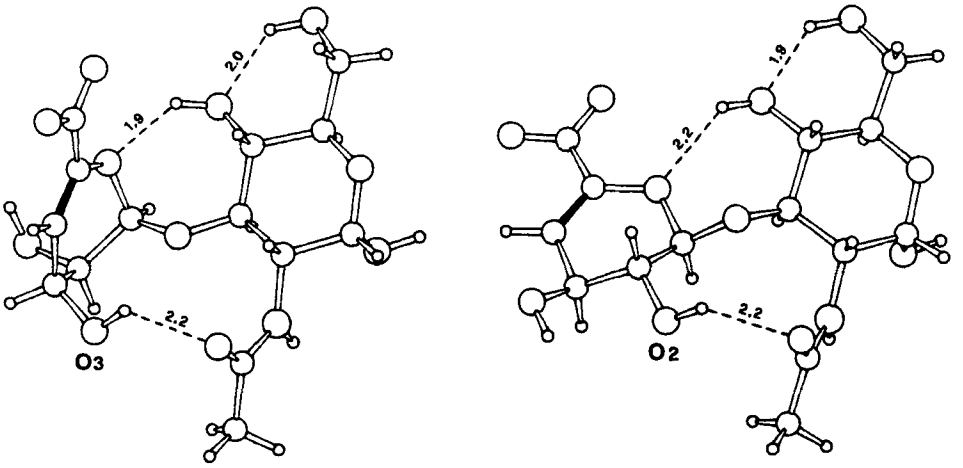


FIG. 3 Comparison between the H-bond networks in the two minimum-energy structures of compound **6**, as obtained with $\varepsilon = 1.5$. Energy of the conformer with uronic ring in the $^2H_1$ form (right) is about 0.5 kcal/mole higher.

populations derived from vicinal coupling constants. The equilibrium is mainly governed by the balance between hydrogen bonding and other electrostatic interactions, which in turn also depend on the solvent (here taken into account only with different values of the dielectric constant), as well as on the dissociation degree of the carboxylic acid group.

Therefore the predicted populations strongly depend on the value of ε chosen to compute the electrostatic energy: while realistic values are computed also with $\varepsilon = 1.$ or $\varepsilon = 3.$, the best agreement with experiment is obtained with the intermediate value of 1.5 (i.e., an rms deviation of 10% in the populations, with a maximum discrepancy of about 20%). The relatively better agreement for the non-sulfated compounds (starred in Table 5), points out the need of revising the charge distribution on $-SO_3^-$ (and possibly on other ionic groups).

The changes in the conformational equilibrium, although semi-quantitatively justified by the energy calculations, cannot easily be ascribed to single major effects. The $^1H_2$ form is stabilized by a (rather distorted) H-bond between H(O3) and the glycosidic oxygen; calculations done on a series of monosaccharides (results are not reported here) indicate that this conformer dominates the population at equilibrium. As for the disaccharides, a number of different H-bonds may be formed, depending on the nature of the sugar at the reducing end, on the conformation of the unsaturated ring, and on the torsional angles at the glycosidic linkage: examples are shown in Figures 3 and 4.

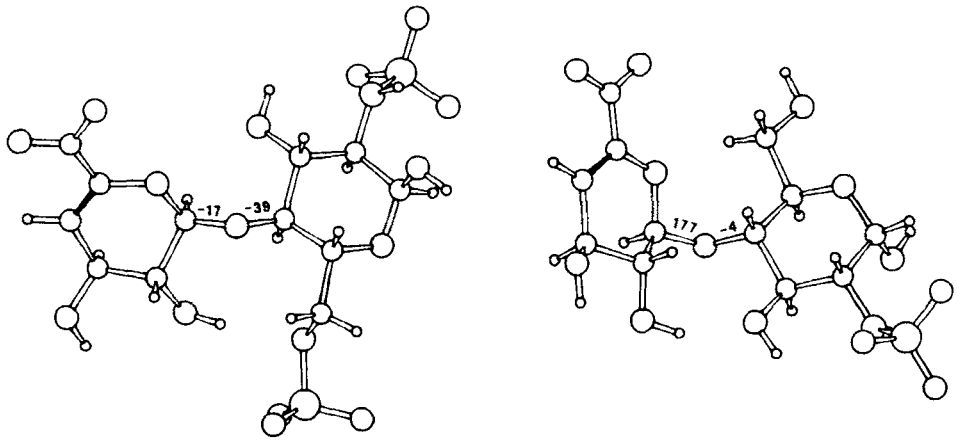


FIG. 4 Minimum-energy structure of compounds **1** (left) and **2**, as obtained with $\varepsilon = 1.5$; the rather unusual values for the torsion angles (φ ; ψ) at the glycosidic linkage are evidenced.

Inspection of the (φ;ψ) maps reveals a general similarity with those computed for disaccharides in our previous works,[25] while noticeable difference is found for compounds **1** and **2**. Their minimum energy conformation (both with the unsaturated ring in $^2H_1$ form), lie near (-17;-39) and (177;-4), respectively, as depicted in Fig. 4; analysis of NOE data may confirm this theoretical result.

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17. Abbreviations used: *ΔUA-GlcNS6S:* 4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-deoxy-2-sulfamido-D-glucopyranose-6-sulfate; *ΔUA-GlcNS:*4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-deoxy-2-sulfamido-D-glucopyranose-6-sulfate; *ΔUA-GlcNAc6S:* 4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-deoxy-2-acetamido-D-glucopyranose-6-sulfate; *ΔUA-GlcNAc (HS):* 4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-deoxy-2-acetamido-D-glucopyranose; *ΔUA2S-GlcNS6S:* 4-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid-2-sulfate)-2-deoxy-2-sulfamido-D-glucopyranose-6-sulfate; *ΔUA-GlcNAc (HA):* 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-deoxy-2-acetamido-D-glucopyranose; *ΔUA-GalNAc:* 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-deoxy-2-acetamido-D-galactopyranose; *ΔUA-GlcNAc4S:* 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-deoxy-2-acetamido-D-galactopyranose-4-sulfate; *ΔUA-GalNac6S:* 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-2-deoxy-2-acetamido-D-galactopyranose-6-sulfate; *ΔUA- GlcNAc-K5:* non reducing terminal disaccharide unit of K5 (*E. coli*) polysaccharide; *ΔUA-GalNAc.ol4S:* 3-*O*-(4-deoxy-α-L-*threo*-hex-4-enopyranosyluronic acid)-D-galactitol-4-sulfate.
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