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Analysis of conformationally restricted models for the (1→6)-branch of asparagine-linked oligosaccharides by n.m.r.-spectroscopy and HSEA calculation*

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ABSTRACT

The conformational preferences of the trisaccharide, β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)-β-D-Glc*p*OR (**1**) have been investigated by n.m.r.-spectroscopy and HSEA calculation. The fixed ω-angle bicyclic analogs **2** and **3**, models for the *gt* and *gg* rotamers, respectively, of **1**, were furthermore examined with the same techniques in an attempt to deduce which of the conformations accessible to **1** was recognized and glycosylated by the enzyme GlcNAc-transferase-V, which acts on a component of the (1→6)-arm of glycoproteins. Only the *gg* bicyclic **3** was found to be reactive with the enzyme and this study concludes, based on conformational analysis, that **1** as well as the natural Asn-linked oligosaccharide are recognized by GlcNAc-transferase-V in only one of the two local minimum energy conformations energetically accessible to these molecules in their *gg* rotamer.

INTRODUCTION

A systematic analysis of the recognition of oligosaccharides by protein receptors requires a detailed knowledge of the conformational properties of the oligosaccharide ligands. The consensus that has been arrived at to date regarding the conformational analysis of oligosaccharides is that the conformational flexibility of pyranosyl glycosidic linkages involving secondary hydroxyl groups, *i.e.*, hydroxyl groups directly attached to sugar rings, is severely restricted as compared to those involving primary alcohols such as (1→6) linkages[1–6]. In the former case of (1→2), (1→3), and (1→4) linkages, some conformational freedom is present about the glycosidic bonds, but >90% of the population of molecules generally resides within about ±20–25° (at normal temperatures) of the so-called φ and ψ angles (see Experimental section for definitions) describing the absolute minimum energy conformer, *i.e.*, in 1–2% of the available conformational space. Furthermore, usually only one minimum energy potential well is significantly populated. The result is that the binding of such oligosaccharides to

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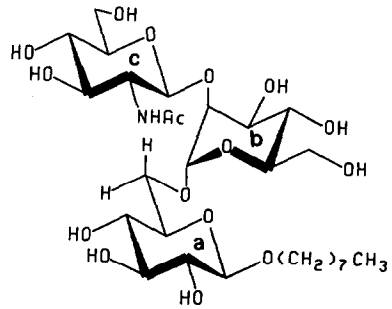
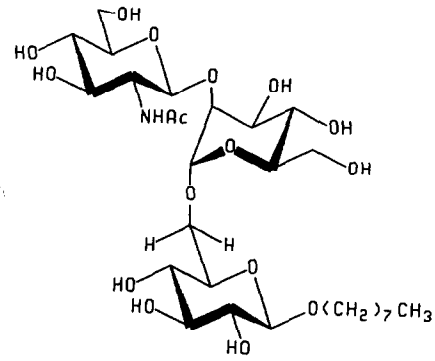
antibodies, lectin, or receptors can be very profitably analyzed by assuming that their three-dimensional shapes are well approximated by the "global" minimum energy conformer. This minimum energy conformer can be estimated by calculation using a variety of computational methods[1-3], the simplest and most widely used of which is the Hard-Sphere-Exo-Anomeric (HSEA) family. The validity of the minimum-energy oligosaccharide conformations derived from HSEA calculations has gained support from high-field n.m.r. measurements[1,2,4].

In contrast, the conformational analysis of glycosides of primary alcohols, such as (1→6)-linked disaccharides, is far more complex for two reasons[5,6]. Firstly, the restraints imposed by steric interactions between the glycosyl and aglycon residues (or glycosyl residues linked at C-1 and O-6) are much reduced, resulting in a greater flexibility in the φ angle (in the order of $\pm 40°$ from the minimum). In addition, the potential energy well is very broad in the ψ dimension, often spanning ± 80–$90°$, and frequently more than one local minimum exists. The oligosaccharides can, therefore, sample more than 10% of the available conformational space with very little difference in energy. Even more important than the decreased steric constraint on such linkages, however, is the imposition of an additional degree of freedom on the linkages, *i.e.*, the possibility for rotation about the C-6–C-5 bonds whose conformation is described by the ω angle (O-6–C-6–C-5–O-5). In most cases studied to date for oligosaccharides containing (1→6)-linked sugar residues, more than one rotamer (*gg*, *gt*, or *tg*) has been found to be energetically accessible and populated both by calculation and n.m.r. data[5-8]. Furthermore, the potential energy surface surrounding each rotamer is very broad and may in turn contain more than one local minimum. The result is that the conformation of a (1→6)-linked oligosaccharide fragment is not only ill-defined with regard to individual C-5–C-6 rotamers, but that more than one local minimum-energy conformer is significantly populated by the molecules for a given rotamer. This means that very few restraints can be put on the conformation and shape of such oligosaccharides. Experimental data on the binding between oligosaccharides and proteins, therefore, defy interpretation at the molecular level.

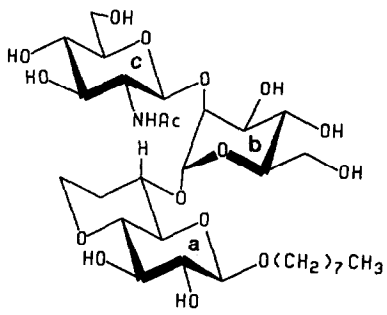
In this paper, we report an examination by n.m.r.-spectroscopy and HSEA calculation of the conformational properties of synthetic oligosaccharides that are models for the flexible (1→6)-arm common to all complex, Asn-linked oligosaccharides. The flexible trisaccharide, β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)-β-D-Glc*p*OR (**1**) has previously been shown to be an acceptor substrate for the enzyme GlcNAc-transferase-V which acts on a component of the (1→6)-arm of glycoproteins. The fixed ω-angle bicyclic analogs, octyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-α-D-mannopyranosyl-(1→6)-4,8-anhydro-7-deoxy-L-*glycero*-β-D-*gluco*-octopyranoside (**2**) and octyl *O*-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-(1→2)-*O*-α-D-mannopyranosyl-(1→6)-4,8-anhydro-7-deoxy-D-*glycero*-β-D-*gluco*-octopyranoside (**3**), models for the *gt* and *gg* rotamers, respectively, of **1** were synthesized[9] in an attempt to deduce which of the conformations accessible to **1** was recognized and glycosylated by this enzyme. Only the *gg* bicyclic structure **3** was found to be reactive with the enzyme.



1(*gt*)



1(*gg*)



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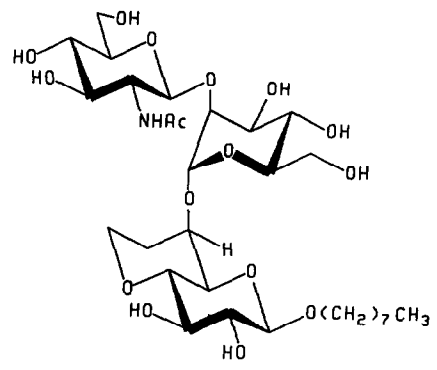

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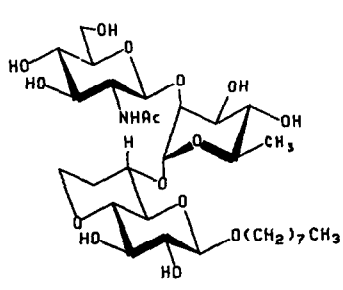
RESULTS AND DISCUSSION

Compounds **1–3**, **6**, and **7** were available from previously published work[9,10]. Compounds **4** and **5** were prepared by similar procedures[11]. Compound **8** was a generous gift from Chembiomed Ltd., Edmonton, Alberta, Canada. The identity of all compounds was confirmed by the present n.m.r. investigations.

The ^{1}H-n.m.r. data obtained at 500 MHz for solutions in D_2O at 27° are given in Tables I and II. The assignments were based on COSY[12], relayed[13], and double-relayed COSY experiments, together with phase-sensitive double-quantum-filtered (DQF) COSY experiments[14]. Similarly, the ^{13}C-n.m.r. data (125.77 MHz) are given in Table III. The assignments were based on heteronuclear correlation spectroscopy[15] with the assigned proton signals and by comparison with data for model compounds[16,17]. Rotating-frame n.O.e. spectroscopy (ROESY) experiments[18] (Table IV) confirmed the intraresidue interactions and assignments, and supported the structures assigned. Comparison of the results from the laboratory-frame nuclear Overhauser enhance-



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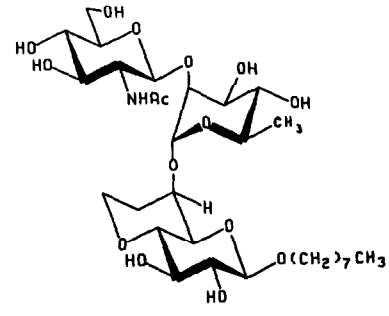

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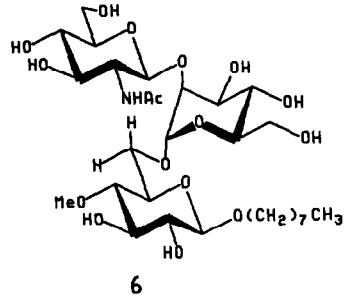

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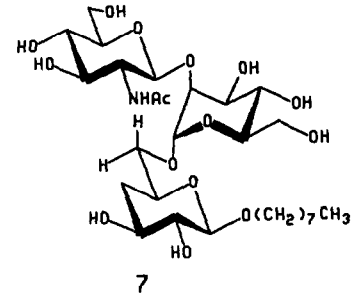

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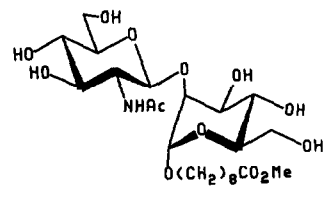

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ments (n.O.e.) experiments (Figs. 1A and B) with the ROESY experiments (Figs. 2A, B, and C) clearly demonstrated the problem associated with laboratory-frame experiments on oligosaccharides of the present size, which have a correlation time long enough so that n.O.e. effects are almost zero. On the other hand, the use of ROESY data for quantitative interpretations is more difficult, but can be used for qualitative interpretations as discussed previously[18,19].

Molecular modelling of the preferred conformations of compounds **1–3** used the

TABLE I

^{1}H-N.m.r. data (δ) for compounds **1–8**

Unit of compound	*Compound*								*Reference*[a]	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	*A*	*B*
β-D-*GlcNAc (c)*										
H-1″	4.55	4.54	4.57	4.53	4.54	4.56	4.54	4.57	4.49	
H-2″	3.69	3.68	3.65	3.69	3.66	3.68	3.69	3.70	3.71	
H-3″	3.53	3.54	3.55	3.53	3.54	3.55	3.52	3.53	3.57	
H-4″	3.44	3.44	3.45	3.45	3.46	3.44	3.44	3.48	3.42	
H-5″	3.40	3.41	3.42	3.41	3.43	3.42	3.40	3.43	3.42	
H-6a″[b]	3.74	3.74	3.75	3.74	3.75	3.77	3.76	3.75	3.75	
H-6b″[c]	3.90	3.90	3.89	3.90	3.89	3.91	3.90	3.90	3.90	
NAc	2.03	2.03	2.03	2.03	2.03	2.05	2.05	2.06	2.03	
α-D-*Man (b)*										
H-1′	4.88	5.02	5.06	4.95	5.00	4.89	4.88	4.87	4.76	
H-2′	4.10	4.02	4.18	4.03	4.19	4.10	4.09	4.06	3.92	
H-3′	3.82	3.79	3.90	3.74	3.86	3.82	3.80	3.78	3.75	
H-4′	3.51	3.57	3.49	3.35	3.35	3.52	3.50	3.51	3.63	
H-5′	3.62	3.84	3.65	3.88	3.71	3.60	3.60	3.57	3.61	
H-6a′[b]	3.63	3.65	3.58	1.23	1.22	3.61	3.62	3.61	3.75	
H-6b′[c]	3.88	3.87	3.87			3.88	3.88	3.88	3.89	
β-D-*GlcOR (a)*										
H-1	4.44	4.44	4.45	4.45	4.44	4.41	4.38		4.37	4.31
H-2	3.24	3.26	3.25	3.27	3.24	3.24	3.14		3.25	3.18
H-3	3.48	3.59	3.51	3.59	3.52	3.54	3.72		3.48	3.77
H-4*e*	3.45	3.09	3.52	3.08	3.50	3.26	2.00		3.37	2.00
H-4*a*							1.51			1.42
H-5	3.54	3.26	3.40	3.26	3.40	3.56	3.82		3.46	3.75
H-6a[b]	3.94					3.96	3.74		3.72	3.64
H-6b[c]	3.72	3.96	4.26	3.86	4.21	3.74	3.66		3.92	3.70
H-7*e*		2.22	1.98	2.20	1.89					
H-7*a*		1.60	1.87	1.60	1.89					
H-8*e*		4.05	3.83	4.04	3.74					
H-8*a*		3.53	3.77	3.52	3.83					
CH_2	3.88	3.87	3.90	3.86	3.89	3.84	3.85	3.53		
CH_2	3.66	3.65	3.66	3.65	3.66	3.64	3.64	3.70		
OCH_3						3.56				

[a]Data for the corresponding methyl glycoside (A) or the corresponding methyl 4-deoxyglycoside (B).
[b]Pro(*R*) proton and [c]Pro(*S*) proton assumed from model compounds and coupling constants.

TABLE II

^{1}H-N.m.r. coupling constants (J, Hz) for compounds **1–8**[a]

Units of compound	*Compound*								*Reference*	
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	*A*	*B*
β-D-*GlcNAc (c)*										
1″,2″	8.4	8.4	8.2	8.4	8.3	8.4	8.4	8.4	8.4	
2″,3″	10.2	10.4	10.3	10.4	10.5	10.0	10.0	10.3	9.6	
3″,4″	9.6			8.4	8.4	9.6	9.6	10.0	9.2	
4″,5″				9.7	9.3	9.8	9.8	10.0	9.2	
5″,6a″	5.4	5.4	5.0	5.2	5.1	5.7	5.7	5.5	4.8	
5″,6b″	2.2	2.1	2.0	2.2	1.9	2.2	2.2	2.2	1.6	
6a″,6b″	12.0	12.2	12.1	12.3	12.3	12.0	12.0	12.2	12.0	
α-D-*Man (b)*										
1′,2′	1.7	1.6	1.8	1.7	1.7	1.7	1.8	1.8	1.6	
2′,3′	3.4	3.4	3.2	3.4	3.4	3.4	3.4	3.4	3.5	
3′,4′	9.6	9.7	9.4	9.8	9.9	9.6	9.5	9.8	9.9	
4′,5′	9.6		9.8	9.5	9.5	9.6	9.5	9.8	9.9	
5′,6a′			7.7	6.2	6.3			7.0	6.4	
5′,6b′			1.7					1.8	2.4	
6a′,6b′			12.2					11.5	11.5	
β-D-*GlcOR (a)*										
1,2	8.0	8.0	8.0	8.0	8.0	8.0	8.0		8.2	7.9
2,3	9.8	9.2	9.2	9.2	9.2	9.8	9.8		9.6	9.5
3,4*e*							5.2			5.1
3,4*a*		9.5		9.4		9.8	10.8		9.6	11.1
4*a*,4*e*							12.5			12.2
4*e*,5							1.8			1.6
4*a*,5		9.5	9.7	9.6	9.6	9.8	10.8		9.8	11.1
5,6a	4.8					4.8	6.0		6.1	7.2
5,6*b*	2.0	9.3	2.4	9.1	2.7	1.8	3.0		2.4	3.1
6a,6b	12.1					11.5	12.3		12.3	12.0
6a,7*e*		5.2								
6a,7*a*		10.9								
7*e*,7*a*		13.1	13.1	13.4						
7*a*,8*e*		5.0	5.1	5.4						
7*e*,8*e*		1.0								
7*a*,8*a*										
7*e*,8*a*										
8*a*,8*e*		12.0	11.7							

[a]See footnotes to Table I.

TABLE III

^{13}C-N.m.r. data (δ) for compounds **1–8**

Unit of compound	*Compound*								*Reference*[a]
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	
β-D-GlcNAc (c)									
C-1″	100.5	100.6	100.1	100.2	99.8	100.8	100.3	100.5	102.3
C-2″	56.4	56.4	56.4	56.2	56.3	56.7	56.2	56.2	56.1
C-3″	74.3	74.4	74.3	74.2	74.1	74.4	74.1	74.2	74.6
C-4″	70.9	70.8	70.8	70.8	70.7	70.9	70.7	70.8	70.9
C-5″	76.8	76.8	76.7	76.7	76.5	77.1	76.6	76.7	76.3
C-6″	61.6	61.6	61.6	61.4	61.4	61.9	61.4	61.5	61.5
NAc	23.0	23.3	23.3	23.1	23.1	23.3	22.8	23.1	23.0
α-D-Man (b)									
C-1′	97.8	94.1	99.3	94.6	99.0	98.8	97.5	97.6	101.9
C-2′	77.2	77.8	77.2	77.5	76.9	77.5	77.0	77.4	71.2
C-3′	70.6	70.6	70.4	70.2	70.1	70.8	70.4	70.6	71.8
C-4′	68.2	67.9	68.3	73.0	73.0	68.5	68.0	68.1	68.0
C-5′	73.8	73.6	74.5	69.8	70.0	74.6	73.6	73.7	73.7
C-6′	62.5	62.4	62.6	17.7	17.6	62.8	62.3	62.3	62.1
β-D-GlcOR (a)									
C-1	103.3	103.2	103.6	103.1	103.4	103.5	103.4		104.0
C-2	74.1	74.7	74.5	74.6	74.3	74.4	75.6		74.1
C-3	77.0	74.3	75.1	74.2	75.0	76.9	70.4		76.8
C-4	70.4	79.5	74.8	79.4	74.6	80.5	46.7		70.4
C-5	75.1	74.7	73.8	74.6	73.7	74.7	71.2		76.8
C-6	66.7	73.3	73.6	74.2	73.4	67.6	69.5		61.8
C-7		23.3	22.9	22.8	22.8				
C-8		66.7	63.6	66.6	63.4				
CH_2	71.8	71.6	71.8	71.7	71.7	71.8	71.5	68.9	
OCH_3						61.5			

[a]Data for the corresponding methyl glycoside.

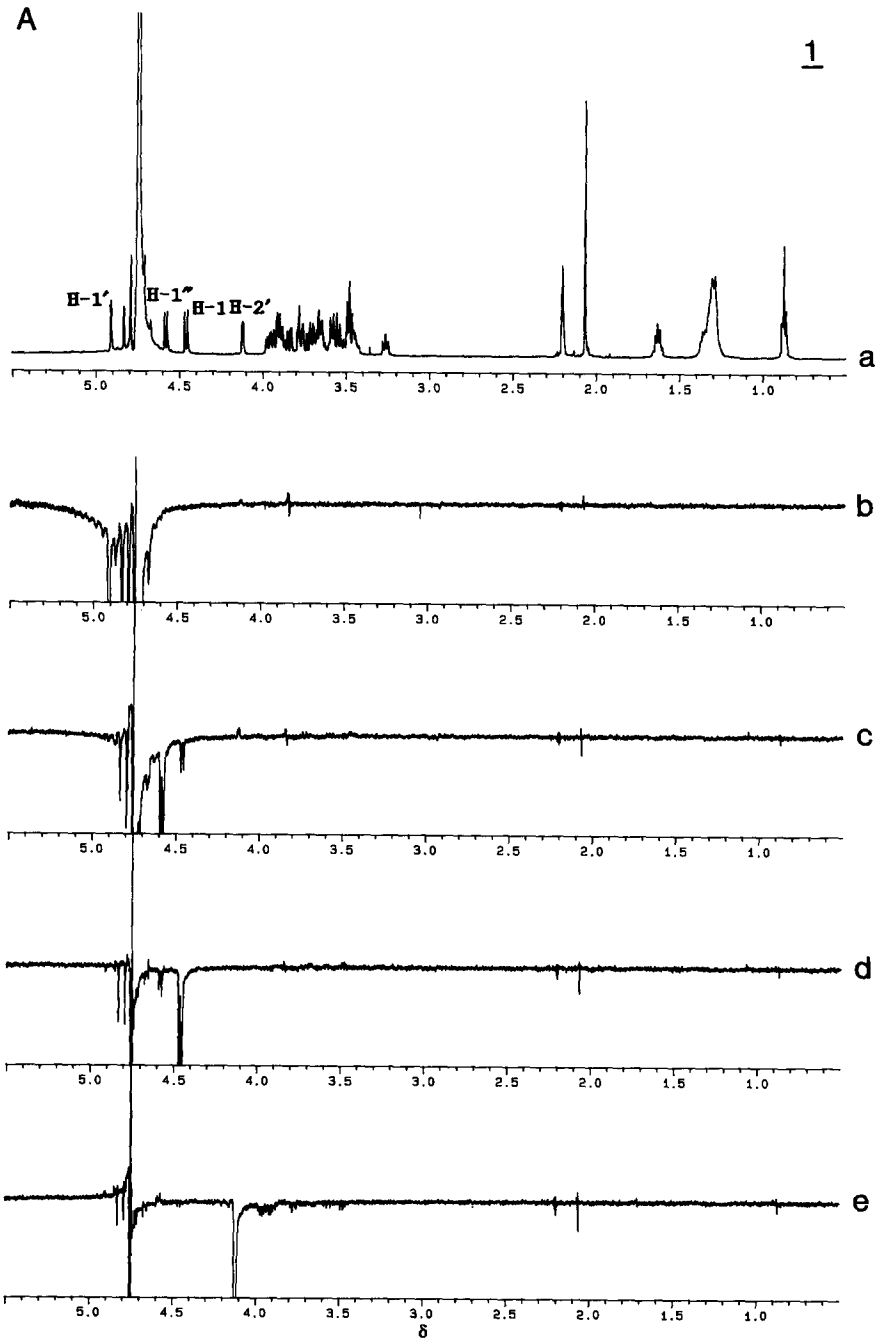

Fig. 1. N.O.e. difference spectra [using a ($^2H_6$)acetone lock] for compounds **1** (A) and **2** (B). respectively, with saturation of the anomeric protons and for **1** also H-2'. The results clearly demonstrate the absence of significant n.O.e.'s.

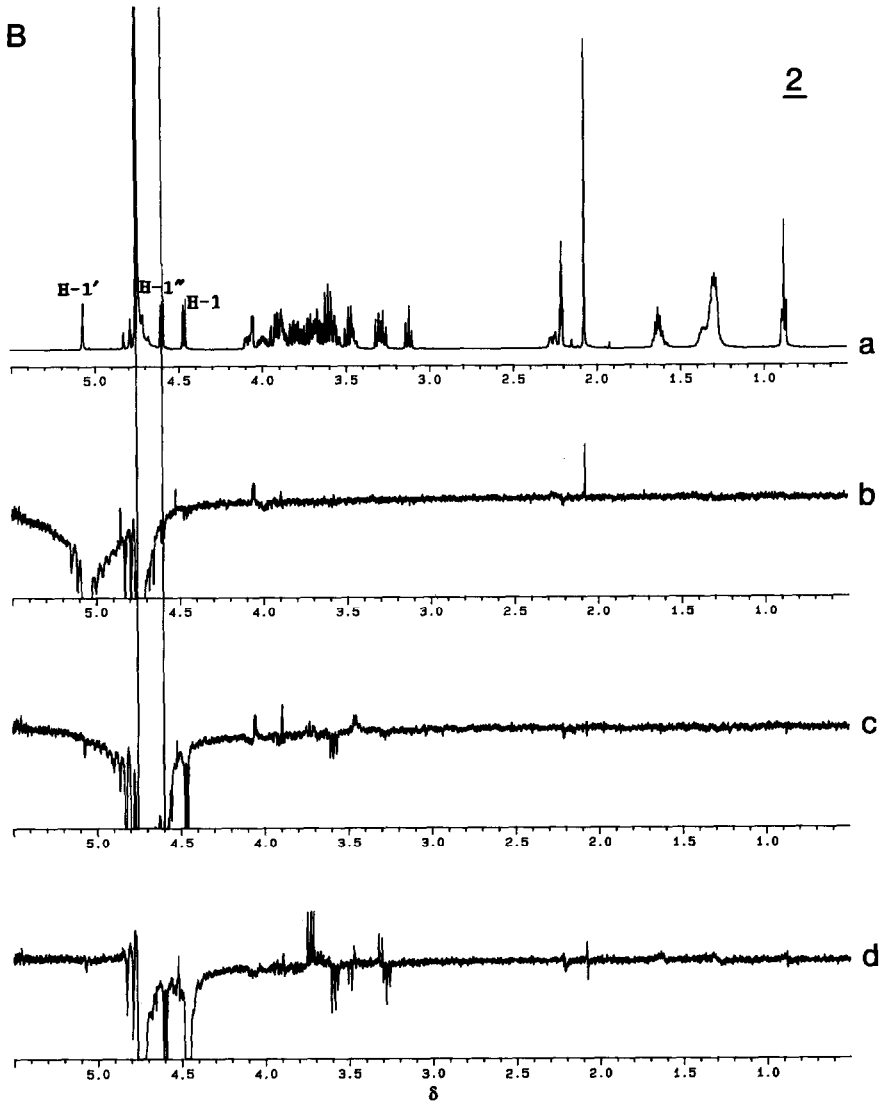
B
2
H-1′
H-1″
H-1
a
b
c
d
5.0
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
δ

TABLE IV

ROESY[a] data for compounds **1–3**

Compound	*Proton saturated*	*Observed n.O.e. (%)*[b]						
		Intraring				*Interring*[c]		
1	H-1	H-2	H-3	H-5		H[d]d	H[e]d	H[f]d
		(0.3[g])	(1.8)	(2.2)		(0.3)	(1.3)	(0.7)
	H-1′	H-2′				H-1″	H-6(*R*)	H-6(*S*)
		(1.6)				(2.8)	(0.6)	(2.3)
	H-1″	H-2″	H-3″	H-5″		H-1′	H-2′	
		(0.6)	(1.9)	(4.6)		(1.6)	(1.4)	
	H-2′	H-1′	H-3′			H-1″		
		(1.3)	(2.1)			(3.8)		
2	H-1	H-2 + 5	H-3	H-4		H[d]d	H[e]d	H[f]d
		(4.4)	(2.0)	(2.5)		(1.3)	(2.1)	(0.2)
	H-1′	H-2′				H-1″	H-6	H-7*e*
		(1.9)				(3.2)	(1.9)	(3.1)
	H-1″	H-2″	H-3″	H-5″		H-1′	H-2′	
		(0.1)	(1.3)	(5.5)		(2.0)	(2.7)	
	H-2′	H-1′	H-3′			H-1″		
		(0.7)	(4.6)			(1.2)		
3	H-1	H-2	H-3	H-5		H[d]d	H[e]d	H[f]d
		([g])	(2.0)	(4.3)		(0.7)	(1.7)	(0.1)
	H-1′	H-2′				H-1″	H-6	
		(2.4)				(3.0)	(1.9)	
	H-1″	H-2″	H-3″	H-5″		H-1′	H-2′	H[d]d
		([g])	(1.9)	(7.6)		(1.9)	(2.0)	(0.1)
	H-6	H-7*e*	H-7*a*	H-5	H-4	H-1′		
		(1.1)	(1.5)	(1.8)	(1.0)	(3.2)		
		H-8*a* + *e*						
		(0.6)						
	H-2′	H-1′	H-3′			H-1″		
		(0.8)	(3.0)			(2.3)		

[a]Measured in % from slices in the ROESY experiment, as described in the Experimental section. [b]Percent in parentheses. [c]Hd denotes proton of the aglycon residue. [d]Signal resonating at δ 3.88 of aglycon residue. [e]Signal resonating at δ 3.66 of aglycon residue. [f]Signal resonating at δ 1.58 of aglycon residue. [g]Dispersion due to spin–spin coupling interaction.

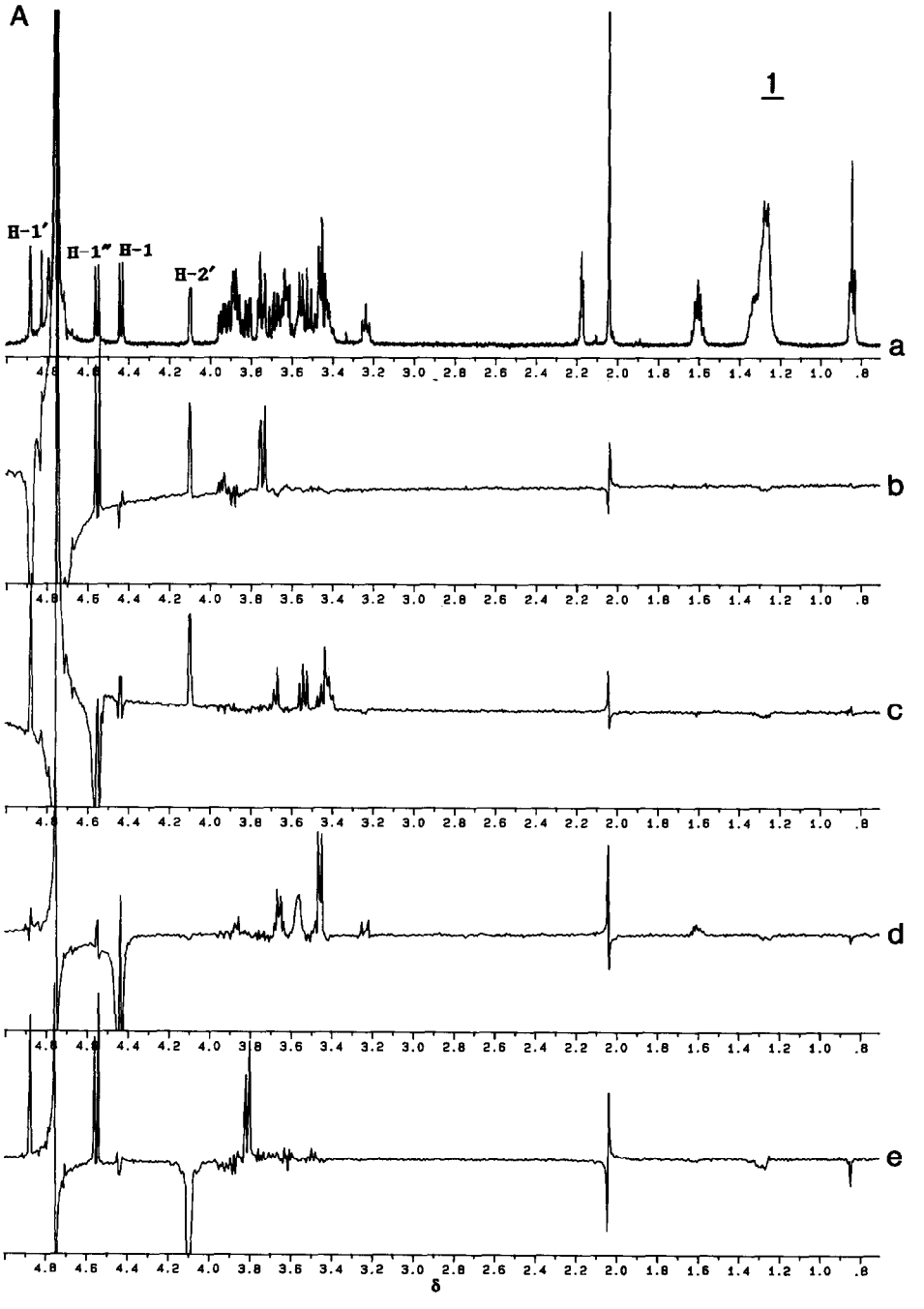

Fig. 2. Rotating frame nuclear Overhauser enhancement (ROESY) experiments [using a ($^2H_6$)acetone lock] for compounds **1** (A), **2** (B), and **3** (C), respectively, with cross sections of the 2D data matrix for anomeric protons and H-2′ in **1** and H-6 in **3**. Assignments and magnitude for the enhancements are given in Table IV.

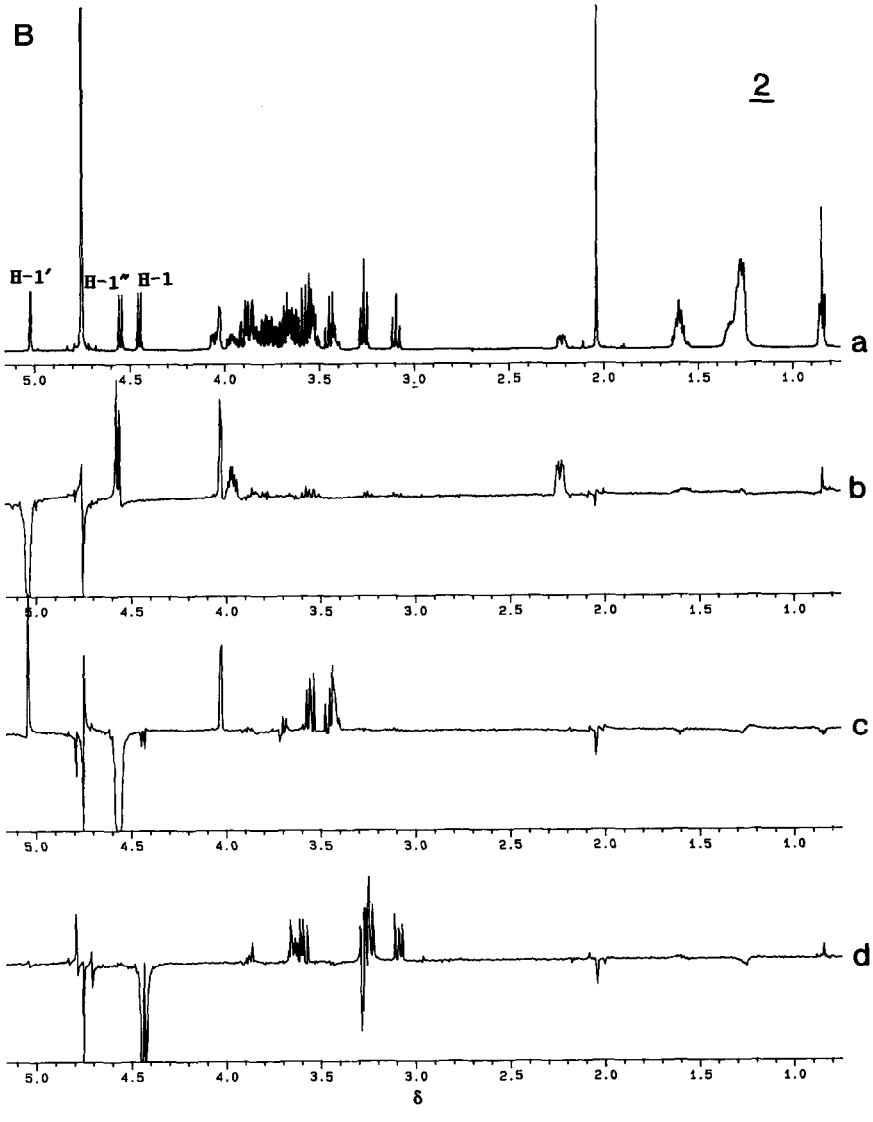

Fig. 2. (continued)

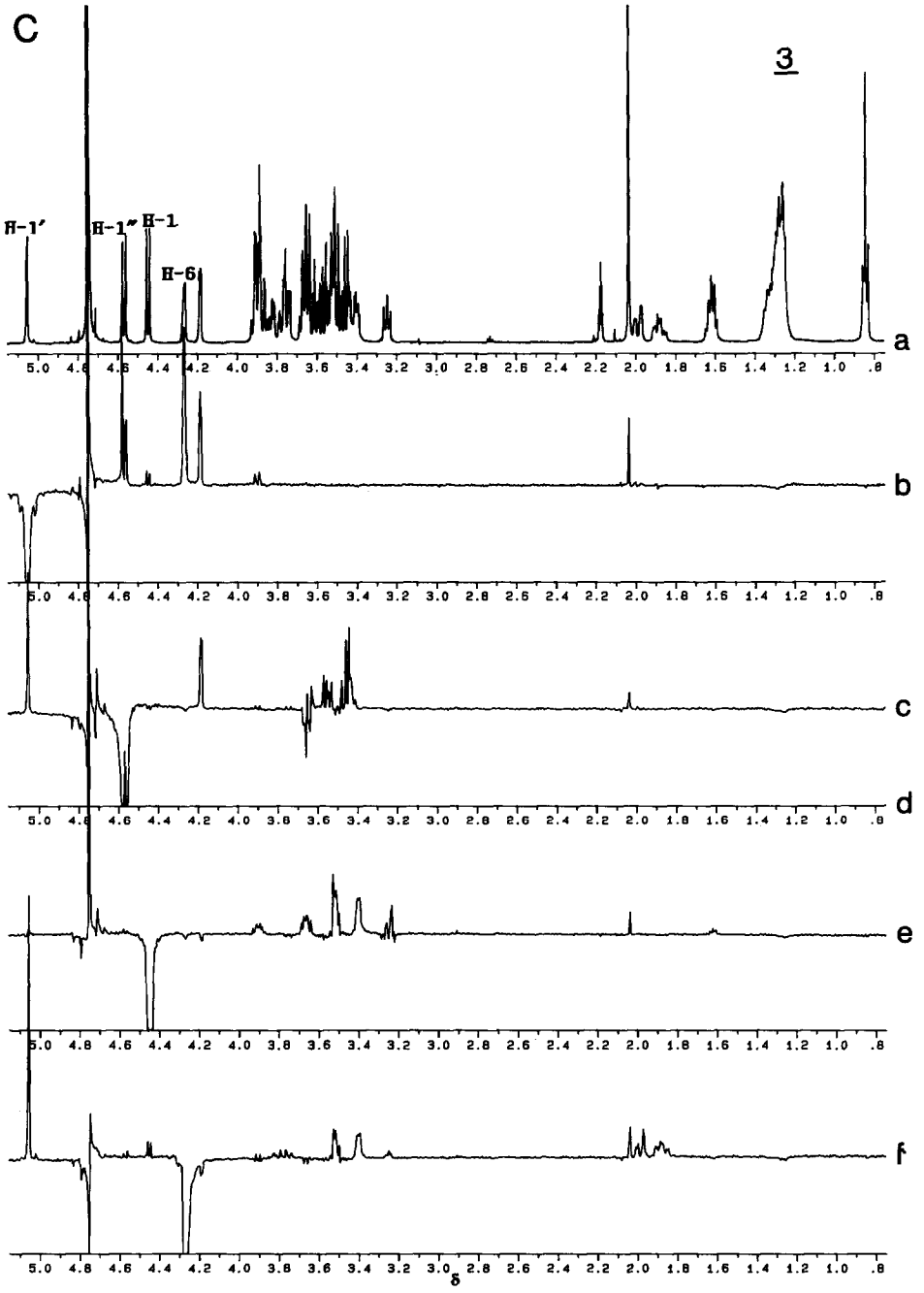

Fig. 2. (continued)

HSEA approach, including ensemble averaging over the whole energy-surface as described recently[20–22]. The torsion angles for the global minima are given in Table V, and selected internuclear proton–proton and proton–oxygen distances in Table VI; these can be correlated with n.O.e. data or specific internuclear shielding effects, as derived from the n.m.r. data presented in Tables I–IV, are given in Table VI. Furthermore, isoenergy-contour diagrams for the two linkages are shown in Fig. 3 to indicate the limited degree of flexibility possible for compounds **2** and **3**, as compared to **1**. The relaxed diagrams (*i.e.*, energy surfaces where variation of the other glycosidic linkages in every gridpoint have been allowed in order to minimize the total energy) show that the influence of the conformation of the β-D-Glc*p*NAc-(1→2)-α-D-Man*p* linkage has some

TABLE V

Minimum energy conformations for compounds **1–3**

Compound	*Omega* (ω)	β-D-GlcNAc*p*-(1→2)-α-D-Man*p*-(1→6)-β-D-"Glc*p*"-OR[a] φ_H,ψ_H	φ_H,ψ	*Energy (kJ/mol)*
1	60,*gt*	54/15	−55/−158	−7.5
1	−60,*gg* Min. 1	52/27	−23/92	−10.0
1	−60,*gg* Min. 2	54/17	−53/−16	−5.6
2	fixed,*gt*	54/17	−50/−146	
3	fixed,*gg*	52/26	−23/88	

[a]"Glc*p*" = Glc*p* for **1** and 4,8-anhydro-7-deoxy-L-(D)-*glycero*-D-*gluco*-octopyranose residue for **2** and **3**, respectively.

TABLE VI

Selected internuclear distances (Å) for compounds **1–3**

Atom pair	*Compound*								
	1 "gt"		**1** "gg"			**2** "gt"		**3** "gg"	
	W. avr.[a]	*Min.*	*W. avr.*[a]	*Min. 1*	*Min. 2*	*W. avr.*[a]	*Min.*	*W. avr.*[a]	*Min.*
H-1″–H-1′	2.57	2.60	2.38	2.28	2.59	2.53	2.54	2.35	2.30
H-1″–H-2′	2.52	2.51	2.60	2.61	2.51	2.52	2.52	2.61	2.58
H-1″–H-1A-d[b]	>4.00	7.42	3.34	2.39	6.55	>4.00	7.89	2.67	2.41
H-1′–H-6	2.60	2.70	2.33	2.27	2.57	2.60	2.59	2.35	2.34
H-1′–H-7*e*						2.54	2.49	>4.00	4.27
H-5′–H-1A-d[b]	3.50	2.39	>4.00	6.65	6.81	2.77	2.37	>4.00	6.69
H-5′–H-1B-d[b]	>4.00	3.93	>4.00	8.00	7.65	3.57	3.30	>4.00	8.15
H-1′–O-5	>4.00	4.65	2.89	2.40	4.48	>4.00	4.62	2.55	2.41
H-2′–O-5″	2.38	2.53	2.49	2.51	2.52	2.52	2.52	2.50	2.53
H-5′–O-5	3.52	2.61	>4.00	5.65	4.53	2.67	2.46	>4.00	5.36
H-4–O-6		4.03		2.54	2.54		4.09		2.55

[a]Distances averaged over the whole energy surface[20–22]. [b]Protons at C-1 of the aglycon residue resonating at δ 3.66 and 3.88, respectively.

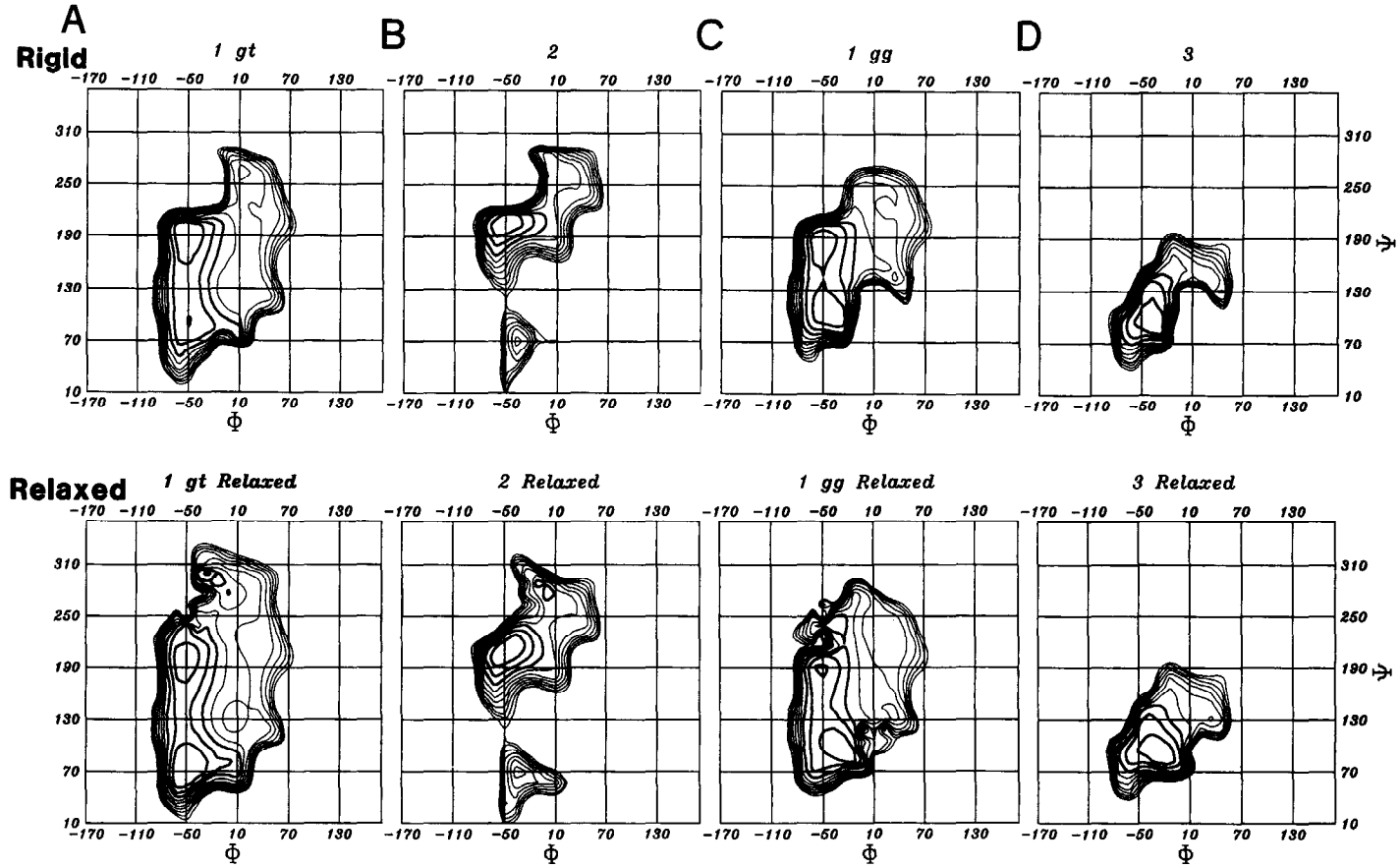

Fig. 3. Isoenergy contour diagrams for rotation around the (1→6)-glycosidic linkage, φ_H and ψ, in compounds **1** (*gt*) (A), **2** (B), **1** (*gg*) (C), and **3** (D), respectively, showing both rigid rotations (top panels) and relaxed [ω Unit (a), φ_H and ψ_H for β-D-Glc*p*NAc-(1→2)-α-D-Man linkages)] rotations (bottom panels).

effect, in that there are conformational restrictions released when this approach is used. It is, however, interesting to note that the distances observed in the global minimum, as compared to the averaged values which require several magnitudes of computer time more to carry out, do not differ substantially. In view of this agreement, a more rigorous analysis using MM2 or similar force fields does not seem to be warranted in this instance.

Inspection of Tables I and II show no significant shift differences for the GlcNAc Unit (c) for compounds **1–7**, when compared to the model monosaccharide or the disaccharide **8**. Furthermore, the proton–proton coupling constants (Table II) confirmed that no significant distortion of the pyranose chair conformations could be observed in the compounds investigated.

For the Man Unit (b), the signal for H-1′ is shifted downfield by ~ 0.1 p.p.m. in compounds **2–5** as compared to **1** and **8** as a consequence of the glycosyl linkage to a secondary OH group. The signals for H-2′ are shifted downfield by ~ 0.1 p.p.m. in compounds **1–8** as compared to the monosaccharide model, which is in agreement with the expected short average distance (2.50 Å, Table VI) between this proton and the ring oxygen atom of Unit (c). However, in compounds **3** and **5**, this downfield shift is increased to ~ 0.25 p.p.m. for compound **1** between the extremes for **2** and **3**. As seen from Table VI, H-2′ of the Man Unit (b) is close to O-5 of Unit (a) (average distance, 2.50 Å), which could account for the observed downfield shift of this proton signal. The conformational model does not, however, offer a plausible explanation for the difference of ~ 0.1 p.p.m. for the signals of H-2′ between compounds **2** and **3**, or **4** and **5**. The signal of H-5′ of the Man Unit (b) is shifted ~ 0.17 p.p.m. downfield in compounds **2** and **4** as compared to **3** and **5**, in excellent agreement with an average distance of this proton to O-5 of the Glc Unit (a) of 2.67 Å and >4.0 Å, respectively.

All the chemical shift differences for H-4, -5, -6, -7, and -8 of the Glc Unit (a) could be related to the fixed orientation of the "*gt*" and "*gt*" orientations in compounds **2** and **3** (or **4** and **5**), respectively, as compared to compound **1** and the model compounds.

Particularly interesting was the observation of a 0.43-p.p.m. chemical shift difference for H-4 in compounds **2** and **3**, which indicated that this chemical shift can be used as a reporter for the relative orientation of the two conformers in equilibrium in studies (*e.g.*, binding studies) where measurements of the H-5 to H-6 coupling constants are not possible owing to either overlap or large linewidths. Based on this observation, it can be seen that the H-4 chemical shift in **1** of δ 3.45, 0.08 p.p.m. lower than in the model monosaccharide (with an almost identical shift for H-2 and H-3), indicated a higher population of the "*gg*" conformer in the 6-substituted trisaccharide **1** than in the monosaccharide. This observation is furthermore reflected in the $J_{5,6(R)}$ coupling constant, which is 4.8 Hz as compared to 6.8 Hz. For recent discussions about the analysis of proton-coupling constants and the orientation of the C-5 hydroxymethyl group, see refs. 7 and 8. The same effect could be applied to the analysis of the rotamer population of the Glc unit in compounds **6** and **7**, and it can be concluded that both the OMe-4 (**6**) and 4-deoxy (**7**) compounds have a higher population of the "*gg*" conformers in the trisaccharides as compared to the monosaccharide models. Furthermore, it should be

noted that the chemical shift of the H-6 pro(*R*) and pro(*S*) protons are changed as compared to the unsubstituted monosaccharides, a general observation for 6-linked oligosaccharides[23], and most likely caused by the short average distance between the ring oxygen atom of the next sugar unit and the H-6 pro(*R*) proton[24]. Finally, the chemical shifts of H-4′ and H-6′ pro(*R*) proton in the Man Unit (b) are shifted upfield by ~ 0.12 p.p.m., when the GlcNAc Unit (c) is present, suggesting a change in the preferred population of the hydroxymethyl group, as also reflected in the $J_{5,6(R)}$ coupling constant, even though it is generally difficult to analyze the spin systems completely owing to chemical-shift overlap.

The n.O.e. data presented in Table IV, obtained from ROESY experiments as described in the Experimental section, are all in excellent agreement with the calculated short average distances also presented in Table VI, in that all calculated short distances are matched with an experimentally observed n.O.e., and vice-versa. The accuracy of ROESY data only allow a qualitative analysis of the agreement between the calculated model and the observed n.m.r. parameters, as performed in the present work. However, such qualitative ROESY data are most useful for the detection of interunit contacts, which are not spin–spin coupled and, therefore, not severely subject to HOHAHA transfer.

Inspection of Table III shows that the signals for the carbon atoms of the GlcNAc(c) and Man(b) Units do not experience significant shifts, as compared to the model compounds or the disaccharide **8**, except for C-4–C-6 in compounds **4** and **5**. However, the C-1 signal in the Man Unit(b) showed quite different shifts in the "*gt*" and "*gg*" analogs **2** and **4**, when compared to **3** and **5**. The former showed an unusual upfield shift of about 4.3 p.p.m., as compared to the reference disaccharide **8**. This observation is in agreement with the dependence of the chemical shift of the anomeric carbon atom from the conformation of the glycosidic linkage[25]. Similarly, C-1 in compound **1** showed a shift between the ones observed for compounds **2** and **3**. For the Glc Unit (a), the most significant carbon-shift differences were observed for C-4 and C-8, with upfield shifts of 4.7 and 3.1 p.p.m., respectively, for these carbon atoms in the "*gg*" compound, as compared to the "*gt*" analog, but in agreement with results expected for the 1,3-diaxial interaction from the OH-6 substituent.

In conclusion, all the experimentally observed carbon and proton chemical-shift differences, coupling constants, and n.O.e. measurements are in good accord with the proposed models (Tables V and VI) derived from the HSEA calculations, but with the conformational flexibility as presented in Fig. 3 where both the 12 kJ/mol limit (bold, *i.e.* >98% of the conformers), together with the 40 kJ/mol limit are shown. Kinetic studies[9] have shown that only the *gg* rotamer of **1** reacts with the enzyme at a significant rate, since the rigid *gg*-bicyclo compound **3** is fully active, whereas the *gt* structure **2** has a barely measurable activity. The trisaccharide **1** populates two different potential energy minima, centered around −55/−170 and −25/−90 for the *gg* rotamer, but only one minimum (−25/−90) is accessible to the rigid analog **3** since a prohibitive steric repulsion results between the underside of the α-D-Man(b) residue and the bicyclo ring in the −55/−170 conformer. Since **1** and **3** have similar kinetic parameters[9], it can be

deduced that the enzyme GlcNAc-transferase-V acts on **1**, and therefore also on the natural Asn-linked oligosaccharide substrates, in one of the local minima ($-25/-90$) present in the *gg*-rotamer.

EXPERIMENTAL

N.m.r. spectroscopy. — Solutions of ~ 2 mg in 0.5 mL of D_2O in 5-mm tubes were used. Spectra were recorded at 500.13 MHz for ^1H and 125.77 MHz for ^{13}C with a Bruker AM-500 spectrometer, and at 27°. The ^1H resonances were measured relative to internal acetone (δ 2.225, DOH at δ 4.75 at 27°), and coupling constants were determined on a first-order basis. The ^{13}C resonances are relative to internal dioxane (δ 67.4).

Homonuclear 2D-n.m.r. spectroscopy was performed with Bruker DISNMRP software, except for the ROESY experiment (see below). Relayed COSY experiments[13] were made with fixed delays of 30 ms and double-relayed COSY experiments with both delays of 30 ms in order to optimize coherence transfer for large couplings[26]. 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 zero filled in the t_1 direction to 512 × 1024 points, treated with a nonshifted sine-bell function in both dimensions, and processed to give magnitude spectra.

The phase-sensitive COSY experiments used double-quantum filtering[14,27] with the Bruker COSYPHDQ microprogram using a fixed delay of 30 ms. These experiments were performed with 512 t_1 increments and a sweep width 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 both dimensions by a shifted sine-bell function before Fourier transformation.

The ^{13}C–^1H correlation experiments[15] were performed with the XHCORRD microprogram using decoupling in the ^1H dimension; 128 t_1 increments of 1200 scans and a size of 2048 points were accumulated. The data matrix was zero filled in the F_1 dimension to 256 × 2048 points before F.t. in the absolute mode, giving a digital resolution of 9.8 Hz in the ^{13}C dimension, and 11.7 Hz in the ^1H dimension.

The ROESY experiments[18,28] used the procedure of Griesinger and Ernst[29] with the spin-lock field surrounded by two hard 90° pulses in order to avoid frequency-dependent effects[19]. The transmitter was used for all hard pulses and the spin-lock field was delivered by the decoupler. The spin-lock field was placed in the middle of the spectrum and a mixing time of 200 ms was used. Quadrature in t_1 was obtained by the hypercomplex method of States *et al.*[30]; 512 t_1 values were recorded with 80 scans each and two dummy scans giving an acquisition time in t_1 of 0.223 s (SW 2300 Hz) and 0.890 s in t_2. The data sets were resolution-enhanced in the t_1 dimension by a shifted sine-bell function and zero filled to 2048 × 2048 data points prior to F.t., thus giving a resolution of 1.1 Hz/point.

HSEA calculations. — These were performed[31] with an IBM PS/2 system, model 80 with a 387 math-coprocessor. The calculation of the ensemble average n.O.e.'s was performed[22] on a TITAN (Ardent Computer Systems) computer as described earlier[20–22]. The angles φ_H and ψ_H are defined as H-1–C-1–O-1–C-X′ and C-1–O-1–C-X–H-X, whereas the linkage to the 6-position of the D-glucose unit ψ is C-1–O-1–C-6′-C-5′ and ω O-6–C-6–C-5–O-5, respectively. The coordinates for the β-D-GlcNAc, α-D-Man, and β-D-Glc units were taken from the X-ray structures or neutron diffraction data, respectively[32–34], and the protons are attached as described[35]. The anhydrooctose units were constructed by bond modification of the aforementioned D-glucose units by use of the molecular modelling program Alchemy[36].

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