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CONFORMATION OF THE PENTASACCHARIDE CORRESPONDING TO THE BINDING SITE OF HEPARIN FOR ANTITHROMBIN III*

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ABSTRACT

The conformation in solution of the pentasaccharide methyl glycoside (A_s–G–A*–I_s–A_M; **1**), which represents the binding site of heparin for Antithrombin III, has been investigated using molecular mechanics and ^{1}H-n.m.r. spectroscopy. The pentasaccharide has a rather rigid (A_s–G–A*) and a more flexible (I_s–A_M) region. A simplified model of **1**, comprising two conformations, corresponding to the $^1C_4$ and the $^2S_0$ forms of the iduronate residue, and modified at the G–A* glycosidic linkage with respect to the energy minimum, reproduces most of the observed ^3J values and n.O.e. enhancements. The possible role in the binding to Antithrombin III of a low-energy conformer, not observed in solution, is discussed.

INTRODUCTION

Recent work on the structure of heparin in relation to its anticoagulant activity indicates that the highly specific interaction with the plasma protein Antithrombin III (AT-III) involves a unique pentasaccharide sequence present in some of the molecules[2]. The corresponding synthetic[3] pentasaccharide methyl glycoside (**1**) binds to AT-III with an affinity the same as that of heparin[3a,4] and, therefore, is an excellent tool for investigating the molecular basis of this interaction. As a first step, we now report on the conformation of **1** in solution.

*A preliminary communication has been published[1].
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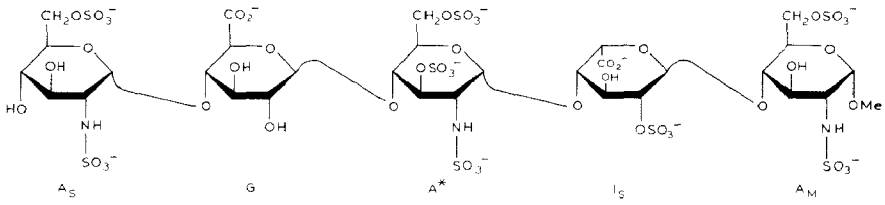

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Theoretical analysis of the conformation of heparin and its oligosaccharides is complex and involves (*a*) the conformation of the iduronate ring, (*b*) the presence of -O-SO_3^- and -NH-SO_3^- groups for which well established stereochemical data and interaction potentials are not readily available, and (*c*) a polyanionic chain, with the associated strong polar interactions, solvent, and counter-ion effects.

Previous approaches to the calculation of the conformation of heparin have been rather crude approximations. Yamaguchi *et al.*[5] interpreted intermediate-angle X-ray scattering data in terms of a statistical-mechanical model based on the approximation of separable conformational energies. Only atoms directly bonded to the pyranose ring were included in the calculation, except for the $-CH_2OH$ group and for the carboxylate group which was replaced by $-CH_2OH$. A similar treatment had been presented by Nagarajan and Rao[6] for the solid-state conformation of heparin, and earlier by Rees[7] for glycosaminoglycans. In these calculations, only rigid $^1C_4$ and $^4C_1$ chair forms of the iduronate ring were considered.

METHODS

Energy calculations. — In considering the high specificity of **1**, any 3D model aimed at clarifying the binding ability must include all groups, if not *all single atoms*.

Resolution of the controversy[8–10] about the conformation of the iduronate ring requires a *fully unrestrained* energy minimization procedure. Such a method was applied first to the sulfated monomer[11] and then to various iduronate-containing oligosaccharides[12]. However, there is a general need for flexible molecular models that consider deformations in bonds and angles in addition to rotations about single bonds, and it is also necessary to assess the extent to which deformations in the side chains or at glycosidic linkages might relieve steric crowding in such structures as **1**.

Molecular-mechanics computations can be combined with experimental techniques in order to define and refine molecular structures, provided that arbitrary assumptions are kept to a minimum by using a consistent force-field without "*ad hoc*" adjustments of parameters. This view is supported by the strategy of the energy calculations for oligosaccharides proposed by Tvaroška and Pérez[13]. Therefore, a search was undertaken for the stable conformers of **1** by energy minimization with respect to *all atomic cartesian co-ordinates*. Hence, in the resulting energy maps, each point represents the minimum value with respect to all

degrees of freedom except for those against which the diagram is drawn.

The force-field used was derived from the well-known MM2 program[14]. The original parameters were modified in order to account for hydrogen bonding. Moreover, the Coulomb energy was computed in the monopole approximation, using an effective dielectric constant of 3 and assuming a total charge of 0.3*e* on each ionic group. Full details of the potentials can be found in refs. 11 and 12. For consistency, the same parameters were used throughout. The approach represents a reasonable answer to (*a*) and (*b*) noted above but gives a relatively crude simplification of the electrostatic energy.

The approach might be improved by using a more refined treatment of the exo-anomeric contribution, taking into account the modifications proposed by Jeffrey and Taylor[15], thus producing a set of potentials close to MM2CARB[13], and by revising the potentials associated with the sulfate group on the basis of new calculations on the crystal packing of model compounds. Much more important is the need for a proper treatment of ionic and solvent (water) interactions*.

With the present approximations, molecular mechanics alone cannot fully elucidate the conformation of **1** in solution. However, combining the results of energy calculations with the experimental data (^3J values and n.O.e. enhancements) will reduce substantially the uncertainties about the molecular structure which is unlikely to involve a unique conformation, but an ensemble average of conformational states.

EXPERIMENTAL

N.m.r. experiments. — A solution of **1** (8 mg) in D_2O (99.96%) was lyophilized and the process was repeated three times. ^{1}H-N.m.r. spectra (500 MHz) were performed at 283 K, using an upgraded Bruker WM-500 spectrometer. Assignments were based on multiple-relay homonuclear correlation experiments[18].

Semi-quantitative n.O.e.'s were derived from a 2D NOESY map[19], obtained with a mixing time of 300 ms. Quantitative values were then obtained from difference spectra after semi-selective inversion of a given multiplet using the decoupler channel.

N.O.e. computations. — The n.O.e. enhancements for a system in conformational equilibrium were computed following the procedure outlined by Cumming and Carver[20]. Thus, when the rate of internal motion (τ_i) is moderate [*i.e.*, higher than the correlation time (τ_c), but shorter than the n.O.e. build-up time (typically near T_1)], the steady-state effect depends on the average value of r_{dk}^{-6} for the conformational equilibrium. The average enhancements $\langle f(d,s) \rangle$ of the n.O.e. on

*Preliminary calculations of this type have been done for a small protein (BPTI)[16] and for Met-enkephalin[17] with a procedure included in the program REFINE.

H_d, due to the saturation of H_s, have been computed by solving the simultaneous equations[21]

$$\langle R_d \rangle \langle f(d,s) \rangle + \sum_{k \neq d} \langle \sigma_{dk} \rangle \langle f(k,s) \rangle = \langle \sigma_{ds} \rangle \qquad (\text{for } d \neq s),$$

where both R_d and σ_{dk} are functions of τ_c, of the internuclear distances r_{dk}, and of the frequency ω_o used; τ_c has been considered as an adjustable parameter and its value was set to 0.61 ns in order to reproduce the overall absolute magnitude of the enhancements. The value of 0.03 s^{-1} for R^s was taken from Brisson and Carver[22]. Since the results led to a simplified model of **1** formed by only two conformers, each corresponding to a different form of iduronate, the elements $\langle \sigma_{ds} \rangle$ of the cross-relaxation matrix have been averaged over the populations of these two states.

RESULTS AND DISCUSSION

Monomers. — (*a*) *Iduronate.* The ring conformation of α-L-idopyranuronate and its 2-sulfate (found in dermatan sulfate and in heparin, respectively) has been a matter of controversy[8,9]. In our force-field study[11], it was shown that three nearly equi-energetic conformations of the ring, namely, the $^1C_4$ and $^4C_1$ chairs and the twist-boat ^2S_o, could contribute to the equilibrium in solution (see the preceding paper[23]).

The interpretation[10,12,23–28] of the ^3J values confirmed this hypothesis. Indeed, all three conformers can be present in the equilibrium, in various proportions, depending on the sulfation of the iduronate moiety and on the nature of the neighbouring residues. The presence of different conformers was subsequently confirmed also by n.O.e. experiments[1,27] (for a review, see the contributions of B. Casu and I. A. Nieduszynski in ref. 29).

Therefore, in the present work, all three ring conformers were taken into consideration. However, as the analysis[24] of the ^3J values has excluded the $^4C_1$ conformer from the solution equilibrium for iduronate residues *inside* a saccharide chain, in particular in heparin and in **1**, more attention has been paid to the other two forms. As far as the side chains of I_s are concerned, rotation about the C-2–O-2 bond shows a single minimum, roughly corresponding to the eclipsed arrangement of sulfur S-2 and H-2. Sulfate groups attached to the pyranose ring always adopt this unique conformation, with deviations up to 30°, depending on the environment. In contrast, HO-3 and similar hydroxyl groups in other residues may assume three distinct conformations, the relative stability of which depends on the form of the ring and on the interactions with neighbouring residues. In general, the most stable arrangements were determined by successive steps of rotation and minimization, but clearly, in solution, there are several conformers. The carboxylate group shows great freedom of rotation, the favored conformation being determined by electrostatic interactions with surrounding polar groups.

(*b*) *2-Amino-2-deoxyglucose*. Di- and tri-sulfated 2-amino-2-deoxyglucose (residues A_s and A*, respectively, in **1**) maintain the standard $^4C_1$ conformation. This result is supported by the n.m.r. data. The presence of the third sulfate group in A* produces deviations in the torsional angles of the ring of $\pm 2°$, and inspection of low-energy structures shows that effects of the same magnitude depend on the position in the chain and on the conformation of the main chain and the side chain. Since the N of the sulfoamino group has a tetrahedral sp^3 configuration, as observed in the crystal structure of model compounds[30], two distinct pyramidal arrangements of the C–NH–S group are possible, so that care is necessary in the optimization of electrostatic or H-bonding interactions. Rotation about the C-2–N-2 bond shows a

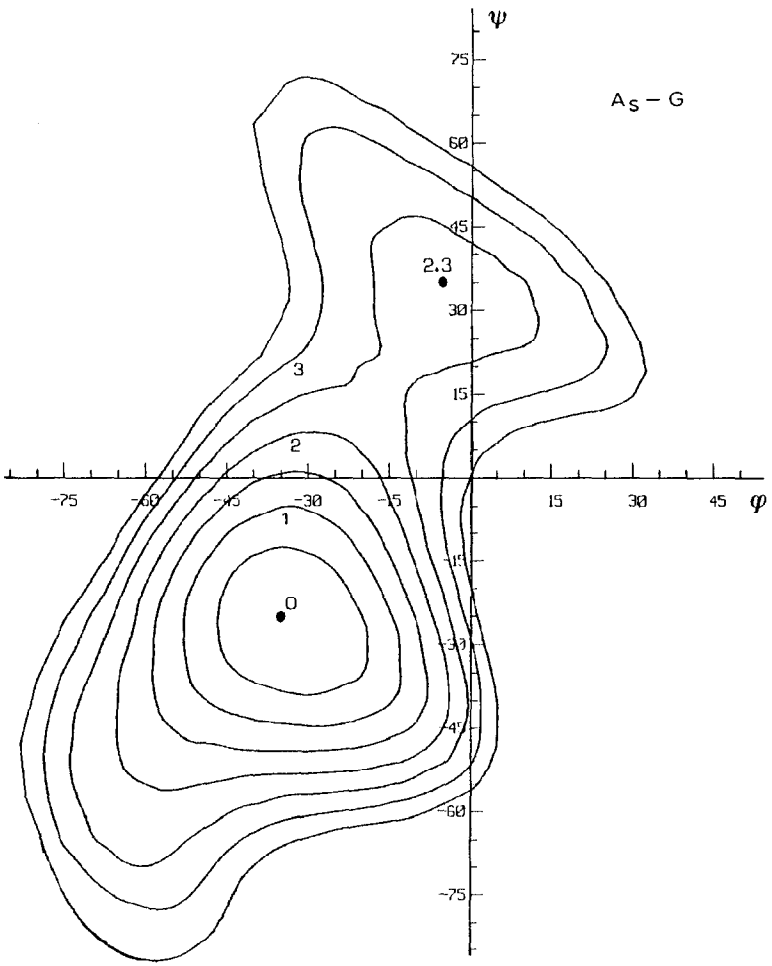

Fig. 1. Map of the energy as a function of the dihedral angles at the glycosidic linkage, φ(H-1–C-1–O-1–C-4) and ψ(C-1–O-1–C-4–H-4), for the A_s–G dimer, obtained by minimization on a 5° grid: distance between levels is 0.5 kcal.mol^{-1}.

single minimum, with the sulfur atom in a quasi-*cis* position with respect to H-2, as for the O–SO_3^- group. The 6-sulfate group has a preference for the *trans* conformation at the C-6–O-6 bond. Of the three possible staggered conformations for the C-5–C-6 bond, the computations on various segments of **1** containing A_s or A* discard conformer (g't) (where O-5–C-5–C-6–O-6 is *trans*), whereas the other two conformers have comparable energies. This conclusion, at least partially, is not consistent with the n.m.r. data, which support the presence of conformer (gg') only.

Disaccharide energy maps. — In order to build up molecular models for **1**, the accessible conformational space was analyzed systematically by computing energy maps for the constituent disaccharides. Preliminary computations had shown that, in spite of the ionic character of **1**, the interactions of first-neighbor residues were overwhelming in determining the preferred conformation. Except for the non-reducing side in the A_s–G pair, the model disaccharides were methylated at each end, in order to simulate the effect of a continuous chain.

After finding one or more minima through unrestrained energy minimization starting from several points in the (φ, ψ) plane, the lowest minimum was refined by optimization of the side-chain arrangements. The result was then used as the starting point for the computation of the map, then checking whether it was the optimal one throughout.

(*a*) *A_s–G.* The map (Fig. 1) shows the global minimum at φ −33°, ψ −26°, with a secondary minimum (φ −5°, ψ 35°) with ΔE 2.3 kcal.mol^{-1}, corresponding to a weak hydrogen bond between N-2–H in A_s and O-2 in G. The (gg') conformation at C-5–C-6 of A_s was maintained for consistency with the small values of $J_{5,6a}$ and $J_{5,6b}$, although the (tg) conformer was nearly equi-energetic.

(*b*) *G–A*.* The map (Fig. 2a) of this disaccharide shows two distinct low-energy regions, with the global minimum at φ 49°, ψ 6°. Comparison with the map (Fig. 2b) computed for the disaccharide G–A_s indicates that addition of the 3-sulfate group has a considerable effect on the conformation of the glycosidic linkage. In fact, a significant portion of the (φ, ψ) space, for negative values of ψ, where the global minimum of G–A_s is located (φ 21°, ψ −46°, ~0.4 kcal.mol^{-1} lower than the minimum at φ 46°, ψ 2°), in G–A* is disallowed due to steric interactions of the 3-sulfate group with the adjacent 2-sulfate group and with O-5 and the carboxylate group of the glucuronate residue. Hence, the energy minimum is shifted towards the negative φ region (φ −4°, ψ −38°, E ~1.3 kcal.mol^{-1} higher than the global minimum). This secondary minimum is unfavored by van der Waals and strain energies, and favored by electrostatic interactions, showing a hydrogen bond in the 2-amino-2-deoxyglucose residue, formed by N-2–H with one of the 3-sulfate oxygens, and a second hydrogen bond between O-2–H of G and O-6 of A*; a longer N-2–H · · · O^- hydrogen bond is associated also with the lower minimum. In contrast, the global G–A_s minimum shows a hydrogen bond between O-5 of G and HO-3 of A_s. Conformations with energies ~1 kcal.mol^{-1} higher are obtained from the above two minima by rotating the HO-3 of G or by flipping N-2–H of A*.

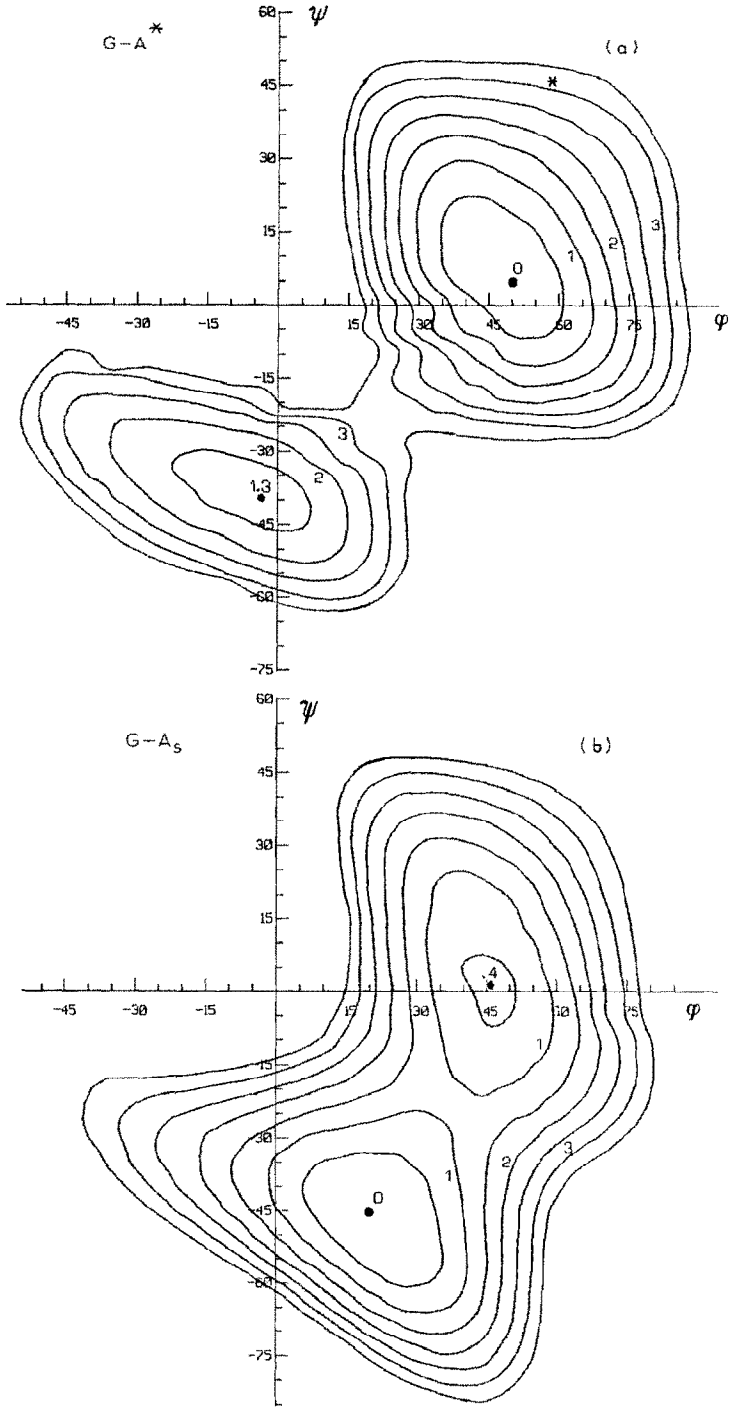

Fig. 2. As in Fig. 1, but for G–A* (a) and G–A_s (b).

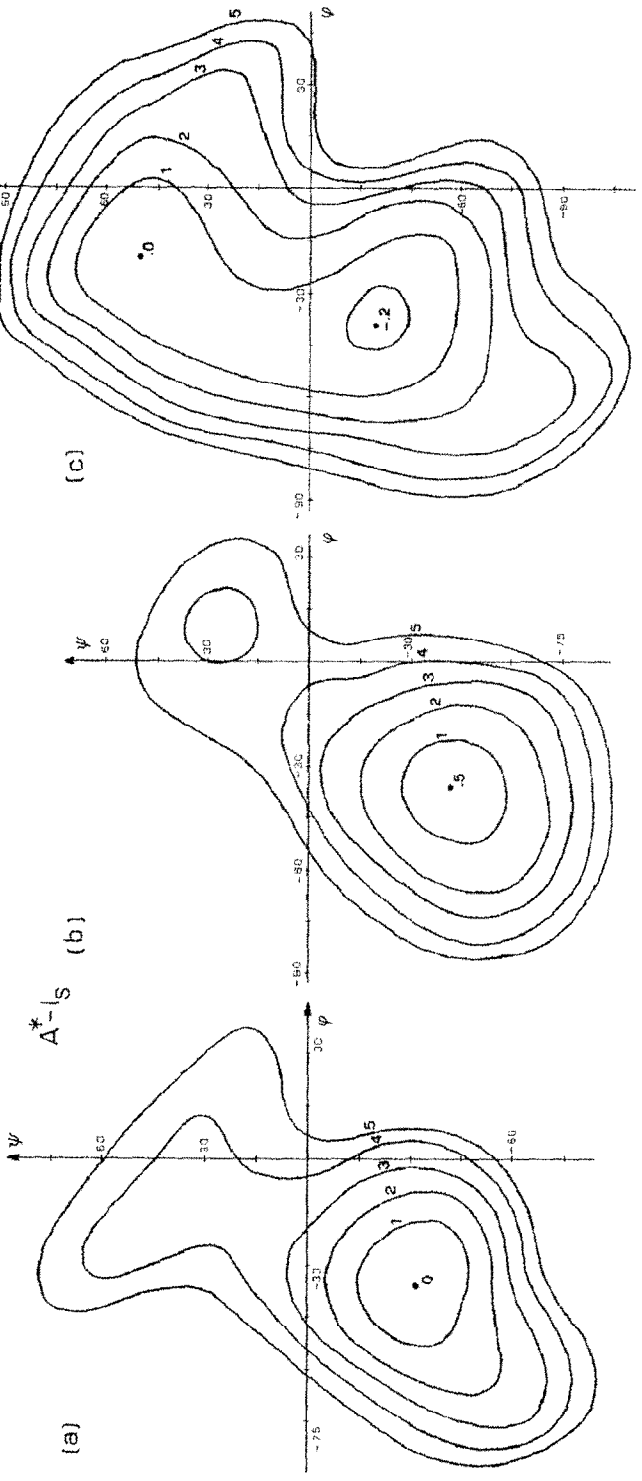

Fig. 3. As in Fig. 1, but for A^*-I_s, for the three possible minimum energy forms of the iduronate ring: $^1C_4$ (a), $^2S_0$ (b), and $^4C_1$ (c).

(*c*) *A*–I$_s$*. Three energy maps (Fig. 3), corresponding to the three allowed conformations of the iduronate ring, were computed for A*–I$_s$. Qualitatively, the maps resemble that for A$_s$–G, which is typical of an axial–equatorial glycosidic linkage, showing the low-energy region for the negative values of φ. The maps differ significantly in the relative stability of the two minima, one in the negative and one in the positive ψ regions. Thus, for the $^1C_4$ and $^2S_0$ forms, the energy difference is even higher than that for A$_s$–G (~3.5 kcal.mol^{-1}), so that only one minimum can be considered with some confidence (respectively at φ −36°, ψ −33° for $^1C_4$, and φ −36°, ψ −42° for $^2S_0$). Conversely, for the $^4C_1$ form, there are two nearly iso-energetic minima at φ −40°, ψ −19° and φ −20°, ψ 50°. The side-chain conformation (gg′) for the C-5–C-6 bond in A* was the most stable, independently of the iduronate ring form, and was kept throughout the map calculations. The only hydrogen bond found for this disaccharide is a weak one between N-2–H of A* and O-2 of I$_s$ in the $^1C_4$ form.

(*d*) *I$_s$–A$_s$*. The three maps (Fig. 4) corresponding to the different forms of the I$_s$ ring were computed first by setting the C-5–C-6 of A$_s$ in the (tg) conformation. In fact, this arrangement appeared to be favored with respect to (gg′) in all the minima found, the difference being small (0.2 kcal.mol^{-1}) when I$_s$ is in the $^2S_0$ form and larger, depending also on the glycosidic linkage conformation, for the $^1C_4$ form. Each of the maps shown in Fig. 4 is similar to that for G–A$_s$, with two nearly iso-energetic minima in the positive φ region. The minima of type I show a hydrogen bond between O-5 of I$_s$ and HO-3 of A$_s$.

The maps for $^1C_4$ and $^2S_0$ were later repeated (see Fig. 5) by taking the C-5–C-6 bond in the (gg′) conformation, which is the only one consistent with the $J_{5,6a}$ and $J_{5,6b}$ values observed for **1**. The effect of rotating the side chain is opposite in the two ring conformers; for the $^1C_4$ form, the interaction of the sulfate groups in position 6 of A$_s$ and in position 2 of I$_s$ greatly restricts the region allowed around minimum II, which is 1.8 kcal.mol^{-1} higher than I. Conversely, for the $^2S_0$ form, the relative stability of the minimum II increases to 1.4 kcal.mol^{-1}. A third minimum was found for this disaccharide in the region around ψ 180° which is usually disallowed (not shown in the Figs.). The relative energy of this conformer is 5.1 kcal.mol^{-1} above the minimum I for $^1C_4$ and only 3.6 kcal.mol^{-1} higher than the minimum II for $^2S_0$.

Energy minima for **1** *and n.O.e. calculations.* — On the basis of the foregoing energy maps, molecular models of **1** were built by taking (φ, ψ) pairs corresponding to disaccharide minima and then minimizing the energy. The assembly of **1** also required a re-optimization of the conformation of the side chains. In a few instances, the final arrangement is not the optimal one for the disaccharides. In general, however, the calculations confirmed the preliminary result that there is little reciprocal influence between non-adjacent residues. Thus, there are small changes in the values of φ and ψ corresponding to the minima; energy differences between conformers obtained for various combinations of disaccharides are nearly additive. A preliminary report on the conformation of **1** has been presented[1]. The

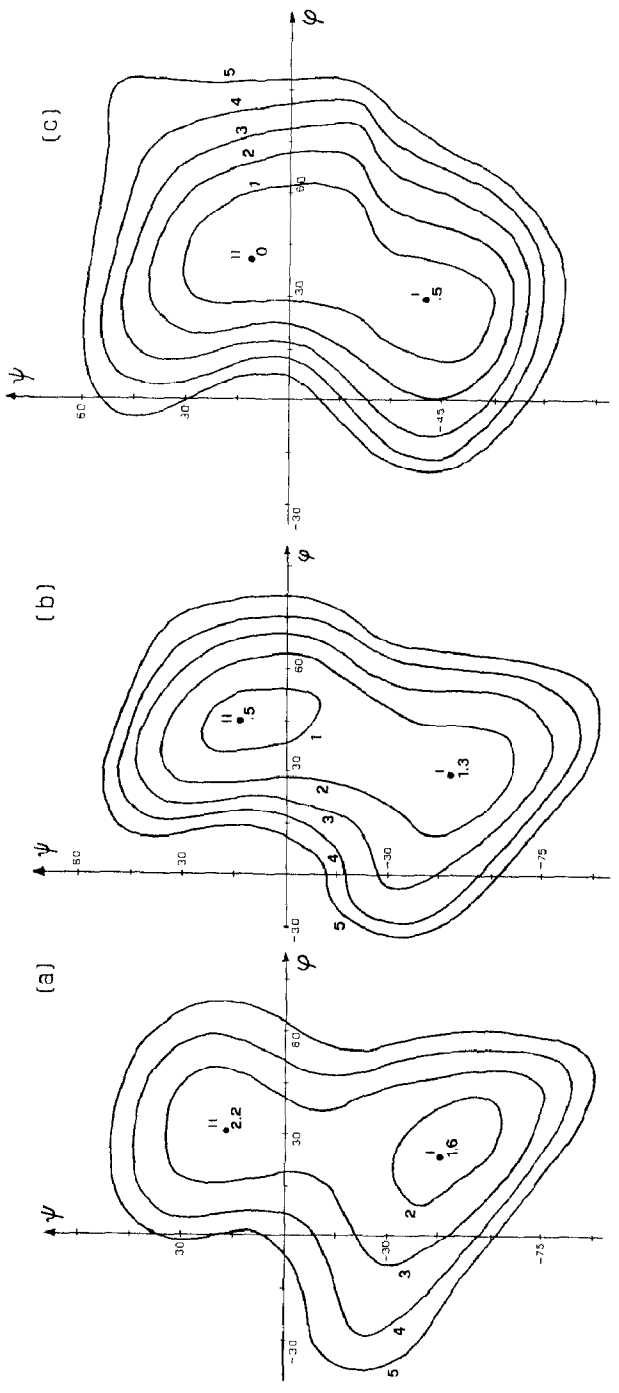

Fig. 4. As in Fig. 3, but for I_s–A_s; the conformation of the C-5–C-6 bond in A_s is (tg).

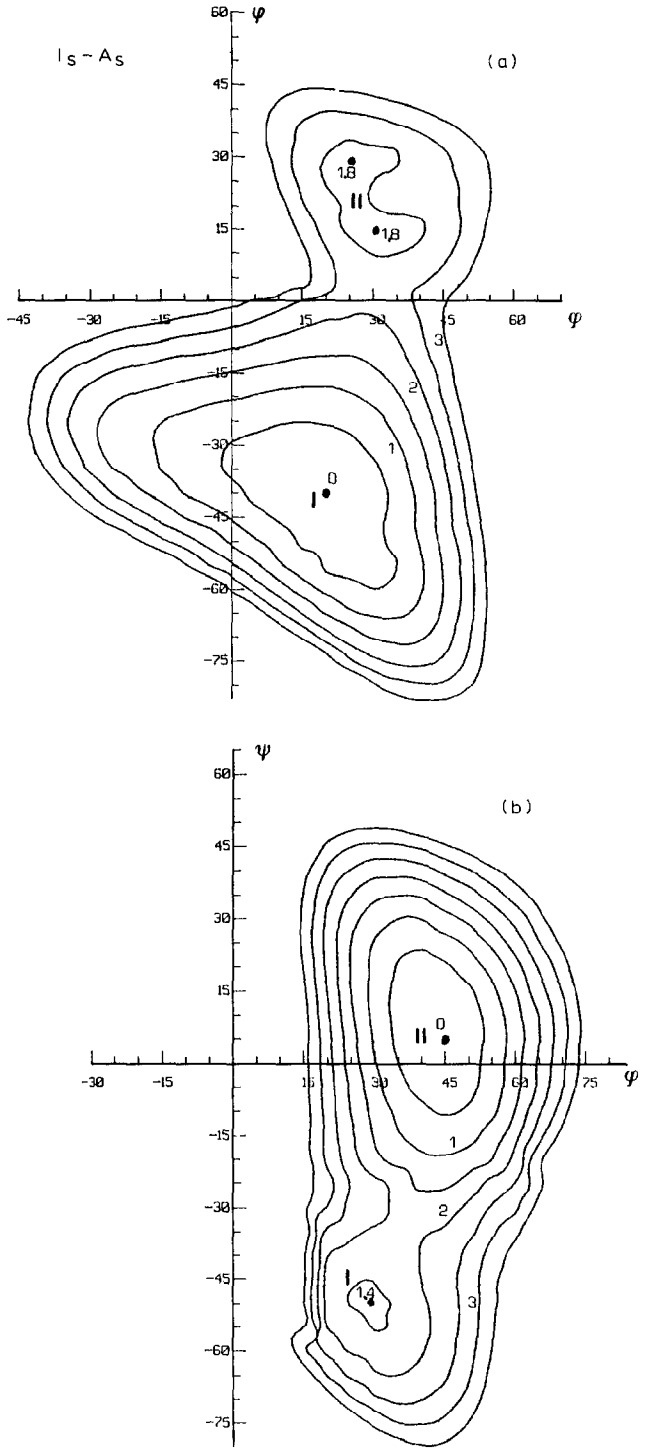

Fig. 5. As in Fig. 4, but with the (gg′) conformation for the C-5–C-6 bond of A_s. The $^4C_1$ conformer of iduronate was not considered. The energy of minimum I in (a) is 1.2 kcal.mol^{-1} higher than minimum II in (b).

structures in Table I and Fig. 2 of that note correspond to a (tg) conformation of C-5–C-6 in A_M, that stands here for the A_s at the reducing end. In the subsequent computations, the (gg′) conformation of such a side chain was adopted for consistency with the ^3J data.

A list of low-energy conformers is given in Table I; this shows the relative energies and the main-chain dihedral angles. Structures corresponding to the $^4C_1$ form of I_s were discarded since they are inconsistent with ^3J values[12]. The most stable conformers for the iduronate $^1C_4$ and $^2S_0$ forms, obtained from the respective lowest minimum for each disaccharide, have similar energies. The alternative conformations for the glycosidic linkages A_s–G, G–A*, and I_s–A_M yield structures with energies higher by 2–3 kcal.mol^{-1}. The effect of confining the C-5–C-6 bond of A_M to conformation (gg′) on the relative stability of minima I and II is larger than at disaccharide level, particularly for the $^1C_4$ form, where the difference is 3 kcal.mol^{-1}. The two most stable conformers are shown in Fig. 6.

In view of these results, an attempt was made to calculate the n.O.e. enhancements for **1**, utilizing only the two lowest energy conformers and following

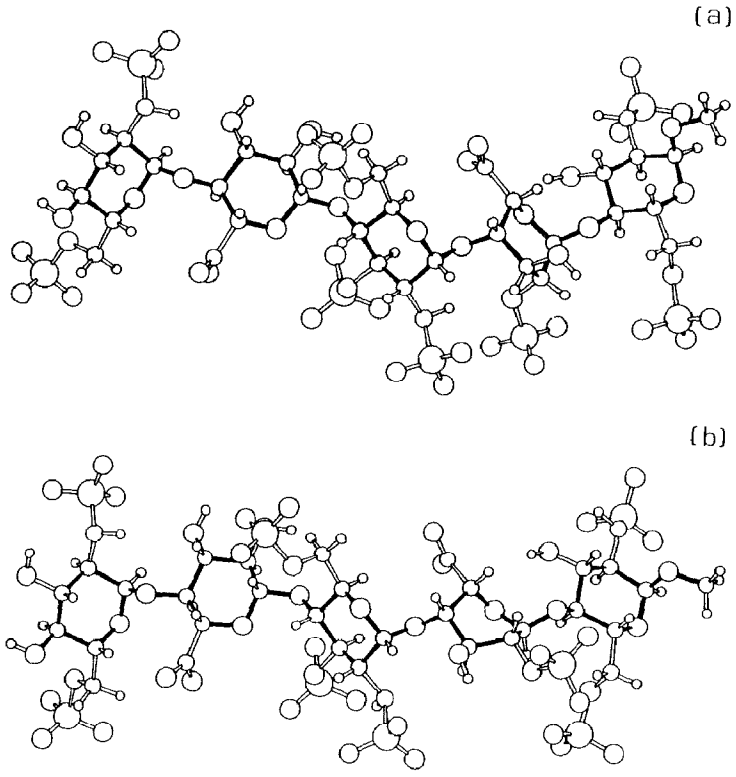

Fig. 6. The two most stable conformers computed for **1**: (a) minimum C1111 (see Table I), corresponding to the $^1C_4$ chair form of I_s, and (b) S1112 ($^2S_0$). For clarity, bonds in the rings and in the glycosidic linkages are darkened.

TABLE I

LOW-ENERGY CONFORMERS OF **1**

Conformer[a]	*Energy* (*kcal.mol*$^{-1}$)	A_s–G (φ_1, ψ_1)	G–A^* (φ_2, ψ_2)	A^*–I_s (φ_3, ψ_3)	I_s–A_M (φ_4, ψ_4)
C 1 1 1	0.00	−32, −27	44, 14	−39, −33	24, −48
S 1 1 2	0.16	−34, −27	47, 6	−35, −42	43, 6
C 1 1 2	2.98	−34, −28	46, 8	−38, −29	32, 22
S 1 1 1	1.85	−34, −27	48, 5	−34, −42	35, −44
C 2 1 1	2.46	−1, 33	46, 12	−39, −32	24, −48
S 2 1 2	2.66	−3, 32	48, 6	−33, −42	43, 5
C 1 2 1	1.66	−34, −29	−16, −36	−37, −32	23, −46
S 1 2 2	2.38	−37, −31	−20, −36	−32, −44	43, 4
C 2 2 1	4.34	−7, 26	−17, −35	−36, −32	23, −46
C 1 1 3	3.29	−31, −27	45, 16	−33, −29	20, 178
S 1 1 3	2.25	−34, −27	47, 7	−31, −42	32, 178
S 1 3 2	5.44	−36, −26	57, 45	−32, −47	42, 7

[a]C and S indicate the $^1C_4$ and $^2S_0$ forms of the iduronate ring; the other indexes refer to the glycosidic linkages in the same order as listed; 1 and 2 correspond to minima I and II in Figs. 1, 2a, and 5; 3 indicates special conformations (see text).

TABLE II

OBSERVED AND COMPUTED N.O.E. ENHANCEMENTS FOR **1**[a]

s	*d*	*Obs. d*	*Calc. d*	*Opt. d*
A_s-1	A_s-2	−16	−14	
A_s-1	G-4	−14	−15	
G-1	G-2	−3	−6	−6
G-1	G-3	−13	−12	−11
G-1	G-5	−14	−17	−15
G-1	A*-4	−8	−14	−7
G-1	A*-5	−6	−1	−4
G-1	A*-6a	−14	−3	−13
G-1	A*-6b	−6	−1	−4
A*-6a	A*-6b	−32	−26	−26
A*-6a	A*-5	−10	−11	−12
A*-6a	A*-4	−4	−3	−4
A*-1	A*-2	−16	−13	
A*-1	I_s-3	−10	−12	
A*-1	I_s-4	−12	−10	
I_s-2	I_s-3	−10	−6	
I_s-2	I_s-4	−10	−4	
I_s-2	I_s-5	−12	−13	
I_s-5	I_s-2	−12	−14	
I_s-5	I_s-4	−12	−11	
I_s-1	I_s-2	−5	−6	
I_s-1	I_s-3	−5	−5	
I_s-1	A_M-4	−6	−15	
A_M-1	A_M-2	−17	−14	
A_M-1	Met	−7	−8	

[a]Optimized values were obtained with constrained minimization of the G–A* glycosidic linkage.

the method described above. Correlations between observed n.O.e.'s and ^3J, such as that mentioned in the preceding paper[23] for one hexasaccharide (*23*), were encouraging in the context of the possible rationalization of the n.O.e.'s observed for **1** on a 500-MHz spectrometer. The n.O.e.'s were averaged by assuming the iduronate conformer population, derived from the ^3J values (*i.e.*, 64% of $^2S_0$)[12]. The computed values are compared with the observed n.O.e.'s in Table II.

In considering the significant interactions associated with the conformation of **1**, there is a good agreement for the linkages A_s–G and A*–I_s. Moreover, the calculations reproduce well the n.O.e. enhancements relative to the interactions within the iduronate ring: the discrepancies for the effects I_s-2→I_s-3 and I_s-2→I_s-4 probably arise from the overestimate of the experimental values due to the overlap of peaks. Thus, the same conformational model explains both the ^3J and the n.O.e. data.

Two points of serious disagreement between experiment and calculations involve the linkages G–A* and I_s–A_M. For G–A*, saturation of proton G-1 affects four protons in A*. The model overestimates the G-1→A*-4 effect, and predicts hardly any enhancement for the side-chain protons. Calculations in which the n.O.e.'s were averaged over the whole (φ, ψ) space for G–A*, using the energies mapped in Fig. 2a, did not account for this discrepancy. A conformational model able to reproduce the four effects consistently was searched for by performing a restrained energy minimization run, where the four distances were kept close to proper values. The compromise structure indicated as S1312 in Table I (and shown in Fig. 7) was found, which corresponds mainly to a rotation by ~40° around the glycosidic bond (O–C-4) of A* (approximately the conformation * in Fig. 2a). Such a rotation brings the side chain -CH_2-O-SO_3^- of the 2-amino-2-deoxyglucose close to the glucuronate ring (see Fig. 8) with a van der Waals energy increase of at least 3 kcal.mol^{-1}. In balancing such a large steric hindrance, interactions must be invoked that are neglected at the present level of calculations, *e.g.*, a salt bridge or solvent interactions. Although the existence of this kind of driving force has yet to be proven, the need of such a modification of the model appears to be required by concurrence of as many as four n.O.e. enhancements. Also, the model with I_s in the $^1C_4$ form can be modified in a similar way.

The model overestimates the I_s-1→A_M-4 effect. Although there are many φ, ψ values which would reproduce the observed n.O.e. enhancement, with energy increase of the order of 1 kcal.mol^{-1}, there is not sufficient information to modify the model uniquely.

Inspection of the present model (see, for example, Fig. 7) shows that, of the anionic groups considered as essential for binding to Antithrombin III[2b,31,32], those belonging to the rather rigid trisaccharide A_s–G–A* (namely, the 6-sulfate of A_s and the *N*- and 3-sulfates of A*), as well as the carboxylate group of G are arranged on the same side of the molecule, whereas the *N*-sulfate on the 2-amino-2-deoxyglucose at the reducing end points in the opposite direction. Thus, rotation around the O–C-4 bond to ~180° would also align this group with the other essential

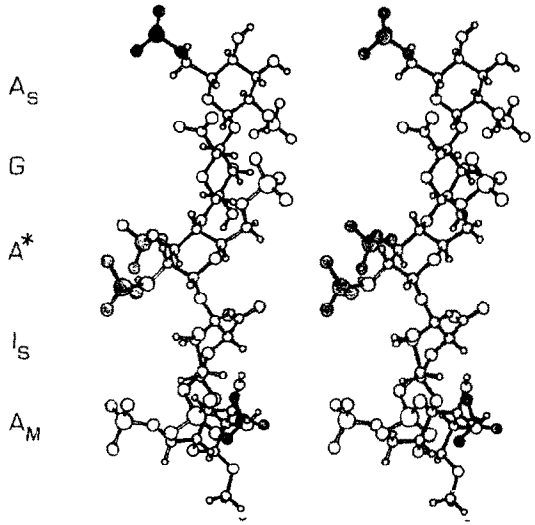

Fig. 7. Stereo-view of the proposed structure S1312 (see Table I), accounting for the observed n.O.e. enhancements[15] (see text). Groups essential for the activity of **1** are shaded.

groups. Since the calculations on I_s–A_s had provided a conformation in the region of $\psi \sim 180°$ with a not exceedingly high energy, such a conformation in **1** was examined. Conformers indicated as C1113 and S1113 in Table I (and shown in Fig. 9) were obtained; particularly for the $^2S_0$ form, the increase in energy is quite modest (~2 kcal.mol^{-1}). This conformation does not appear in the equilibrium in solution, as it would imply a very strong I_s-1→A_M-3 n.O.e., which is not observed. Although two distinct sub-regions of binding on AT-III may exist, as has been suggested recently[33], a two-step process could also be conceived, whereby AT-III first binds to A_s–G–A* only, and then conformational changes occur both in the

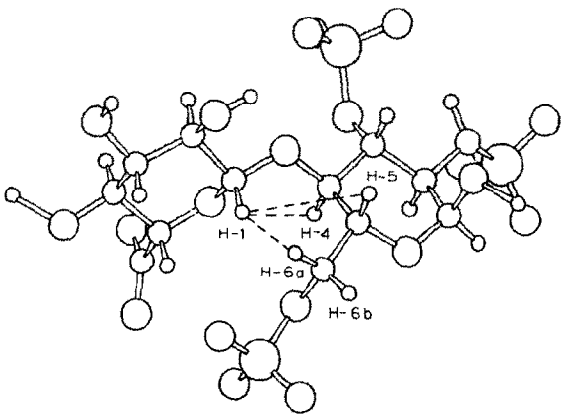

Fig. 8. G–A* segment from the S1312 structure; interactions marked with dotted lines are discussed in the text.

protein and in **1**, such as the "choice" of one of the ring forms of I_s and the adoption of the third conformation at the I_s–A_M linkage.

All molecular structures were drawn by using the SCHAKAL program[34], as adapted (by M.R.) for the local HP-9000 computer.

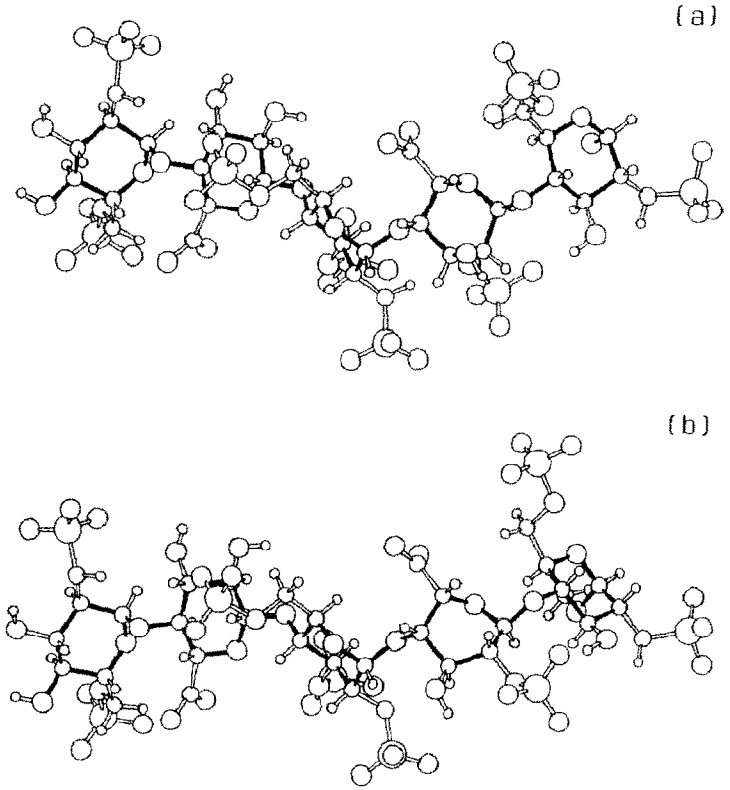

Fig. 9. Structures of **1**, corresponding to an alternative conformation of the I_s–A_M glycosidic linkage (minimum III): C1113 (a), and S1113 (b).

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