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Note

The systematic use of negative nuclear Overhauser constraints in the determination of oligosaccharide conformations: application to sialyl-Lewis X

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High-resolution nuclear magnetic resonance spectroscopy is a very powerful tool for determination of the conformations of biological macromolecules, especially proteins and nucleic acids, in solution[1]. For the conformational analysis of oligosaccharides, each monosaccharide ring is usually assumed to have a single rigid conformation independent of the rest of the oligosaccharide. Thus, the problem of determining the solution conformation of an oligosaccharide reduces to that of determining the torsion angles about the glycosidic linkages, ϕ, ψ, and, for 6-linked sugars, ω (ϕ = H-1–C-1–O-1–C-X, ψ = C-1–O-1–C-X–H-X, and ω = O-6–C-6–C-5–H-5). For sialic acid linkages, referred to later, the notation is slightly different as there is no H-1 proton and the linkage is from C-2; thus, ϕ = C-1–C-2–O-2–C-X and ψ = C-2–O-2–C-X–H-X.

There are two types of NMR parameter that can be used to obtain conformational information: 3-bond coupling constants, either homonuclear or heteronuclear, which are related directly to the torsion angle between the two nuclei[2], and nuclear Overhauser effects (NOEs or ROEs) which are related to the distance between the two nuclei (r) and the correlation time (τ_c) used to describe their motion relative to each other[3]. However, there are a number of limitations when applying these approaches to the conformational study of oligosaccharides. (*1*) The use of heteronuclear coupling constants to characterise glycosidic linkages is often precluded by the small amount of sample present and the inability to obtain isotopically enriched samples. (*2*) It is often necessary to use ROEs instead of NOEs because of the correlation times typical of small oligosaccharides, and these cannot be easily interpreted in terms of accurate distances[4]. (*3*) The number of inter-residue NOEs or ROEs observed is usually very small, two or three NOEs

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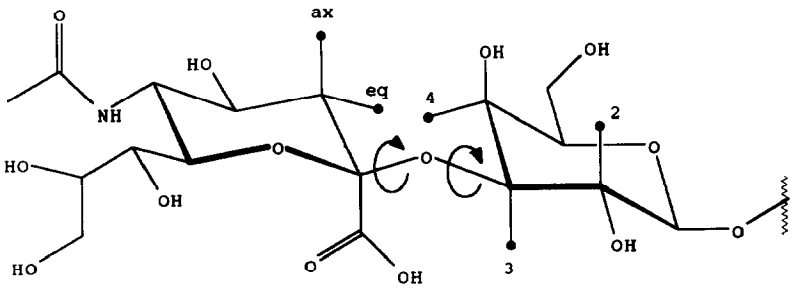

Fig. 1. Schematic diagram of the α-NeuAc-(2 → 3)-Gal linkage. The H-3*ax* and H-3*eq* protons of the NeuAc are labelled *ax* and *eq*, respectively. The H-2, H-3, and H-4 protons of the Gal are labelled 2, 3, and 4, respectively.

per linkage; thus, the problem of calculating torsion angles is not over-determined as it is for proteins[5]. (*4*) Oligosaccharides may exist in solution in multiple conformations in fast exchange. Thus, only average NMR parameters are measured and interpreting these in terms of a single conformation may be misleading.

In order to obtain a better picture of an oligosaccharide in solution, maximum use must be made of the conformational constraints available from the experimental data. This paper discusses the systematic use of all data available from NOE or ROE experiments, with reference to the characterisation of the α-NeuAc-(2 → 3)-Gal linkage in sialyl-Lewis X (Fig. 1).

EXPERIMENTAL AND RESULTS

Sialyl-Lewis X was supplied by Oxford Glycosystems Ltd. Full details of sample preparation and spectral analysis will be published elsewhere. A two-dimensional ROESY[6] spectrum was recorded on a Bruker AM600 at 30 °C and with an 80-ms mixing time.

Fig. 2 shows cross-sections of the ROESY at the chemical shifts of NeuAc H-3*eq* (Fig. 2a) and H-3*ax* (Fig. 2b) resonances, phased to give a negative diagonal. As can be seen, inter-residue correlations are only observed between NeuAc H-3*ax* and Gal H-3 and H-4. It is very difficult to obtain accurate distance constraints from ROE cross-peak intensities because of Hartmann–Hahn exchange[4] and so these ROEs have only been quantified in terms of very loose distance constraints (3.0 ± 0.5 Å for NeuAc H-3*ax* to Gal H-3 and 3.5 ± 0.5 Å for NeuAc H-3*ax* to Gal H-4). The errors reported for these values are estimates, for use in Figs. 3–5, and not based on an analysis of the ROE data or the possible Hartmann–Hahn exchange pathways.

DISCUSSION

Systematic use of positive NOE constraints.—The systematic use of positive NOE information has been well documented[5,7]. From the NOE or ROE intensity, a

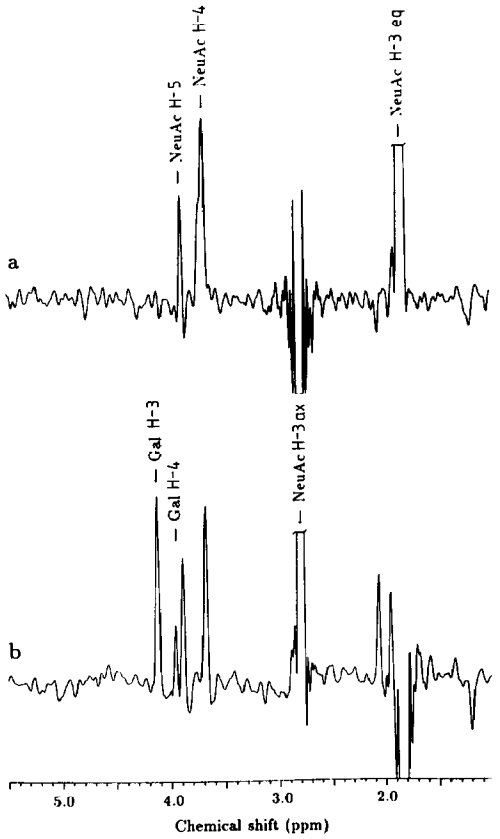

Fig. 2. Cross-sections through the two-dimensional ROESY spectrum of sialyl-Lewis X at the chemical shifts of (a) NeuAc H-3*eq* and (b) NeuAc H-3*ax*, recorded at 30 °C with a mixing time of 80 ms. The spectrum is phased to give a negative diagonal (positive ROE peaks).

distance constraint between two protons on adjacent residues can be calculated. Given the ring geometry for each residue, the distance between any pair of protons can be calculated as a function of the torsion angles about the glycosidic linkage and the region of conformational space consistent with the distance constraint determined. Plots for several different constraints can then be overlaid[5].

Fig. 3a shows the region of torsion angle space (shaded) consistent with the observed ROE from NeuAc H-3*ax* to Gal H-3. Fig. 3b shows the overlay plot for the two observed ROEs, the shaded areas being those consistent with both. As can be seen, there is more than one area of torsion angle space that satisfies both constraints.

Systematic use of negative NOE constraints.—The number of conformational constraints that can be obtained can be increased significantly by using the negative information obtained in NOE or ROE experiments, i.e., the absence of an NOE or ROE indicating a long distance between two nuclei. Negative NOE constraints have occasionally been used in an ad hoc fashion, but are only ever

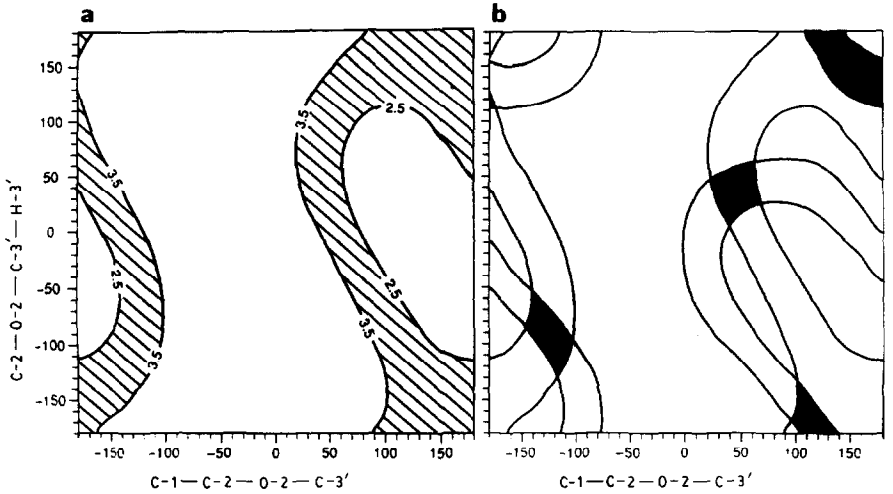

Fig. 3. (a) Torsion angle map for the α-NeuAc-(2 → 3)-Gal linkage, the shaded areas giving the regions with the NeuAc H-3*ax* to Gal H-3 distance between 2.5 and 3.5 Å (consistent with the observed ROE). (b) Overlaid torsion angle map for the α-NeuAc-(2 → 3)-Gal linkage, the shaded areas giving the regions with the NeuAc H-3*ax* to Gal H-3 distance between 2.5 and 3.5 Å and NeuAc H-3*ax* to Gal H-4 distance between 3.0 and 4.0 Å (consistent with the observed ROEs).

included in a systematic way when a full simulation of a NOESY spectrum is performed. One of the procedures adopted for DNA[8] and protein[9] structure refinement is to generate an initial structure using positive NOE constraints, determine magnetisation cross-relaxation and leakage rates from an empirical fit to NOE build-up curves, and use these together with the initial structure to back-calculate the NOESY spectrum. Negative NOE constraints are then included, if necessary, at the refinement stage, but the cross-relaxation rates have to be estimated. This approach is often not viable for oligosaccharides because the starting structure based on positive NOE constraints is not good enough, thus the requirement to obtain as many initial conformational constraints as possible.

The use of negative NOE or ROE constraints has to be treated with care, as the absence of an NOE or ROE can result from either the distance between the nuclei being too great or the correlation time between the nuclei leading to a small NOE or ROE or, in some circumstances, a zero NOE. This ambiguity is a particular problem in the study of biological macromolecules where a large range of correlation times can occur. For a glycosidic linkage, the rings either side of the linkage are assumed to be rigid. The correlation time between two protons on opposite sides of the glycosidic linkage is determined by the overall tumbling of the molecule in solution and by the internal motion about the ϕ, ψ angles of the linkage. Thus, the correlation times between any pair of protons on opposite sides of a given linkage will be similar. In this case, both the presence of one NOE and the absence of another can be interpreted in terms of valid conformational

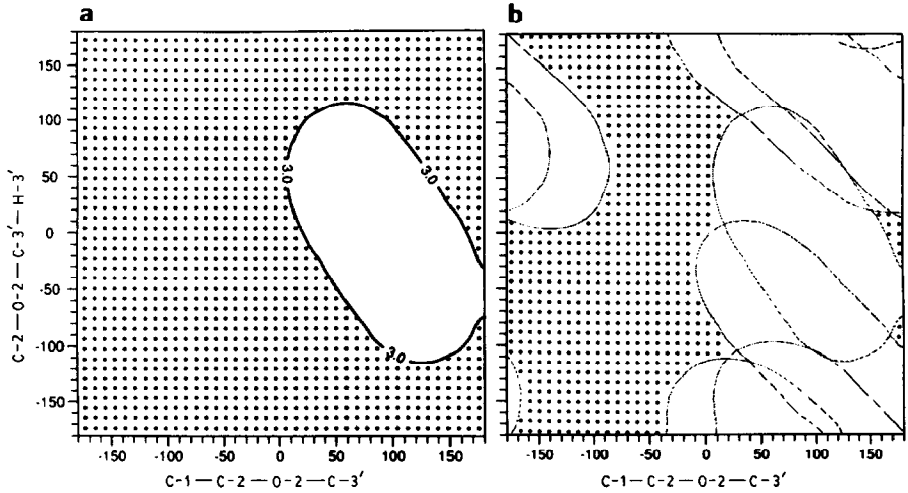

Fig. 4. (a) Torsion angle map for the α-NeuAc-(2 → 3)-Gal linkage, the shaded area giving the region with the NeuAc H-3*eq* to Gal H-3 distance greater than 3.0 Å (consistent with the absence of an ROE). (b) Overlaid torsion angle map for the α-NeuAc-(2 → 3)-Gal linkage, the shaded areas giving the regions with the NeuAc H-3*ax* to Gal H-2 and NeuAc H-3*eq* to Gal H-2, H-3, and H-4 distances greater than 3.0 Å (consistent with the absent ROEs).

constraints. The complete absence of NOEs cannot be treated as a conformational constraint unless an alternative method of estimating the correlation time is employed.

Negative NOE or ROE constraints can be included systematically in the conformational analysis in a similar way to positive NOE or ROE constraints, this time giving a map of excluded conformational space for each linkage.

For the α-NeuAc-(2 → 3)-Gal linkage, the absence of an ROE was interpreted as a minimum distance constraint of 3 Å. Again this is a very loose constraint (the longest distance intra-residue ROE observed is just under 5 Å). Fig. 4a shows the region of torsion angle space (unshaded) excluded by the absence of a NeuAc H-3*eq* to Gal H-3 ROE. This appears to be a very poor constraint compared to those obtained from the positive information. Fig. 4b shows the overlay plot for all the negative ROE constraints. Because of the large number of negative constraints, this eliminates a large area of torsion angle space.

Fig. 5 shows the overlay of the positive and negative constraint plots. Only one region (dark shading) of torsion angle space for this linkage is consistent with all the available ROE data.

Interpretation of NOE / ROE constraint maps.—Given the data presented in Fig. 5, a single conformation for the α-NeuAc-(2 → 3)-Gal linkage ($\phi = -130 \pm 20$, $\psi = -95 \pm 30$) can be used to explain all the NMR data. However, this is only proof for the existence of that conformer if the possibility of multiple conformations in fast exchange can be eliminated (as is assumed in protein structure determination). In this case, there is an important qualitative difference between

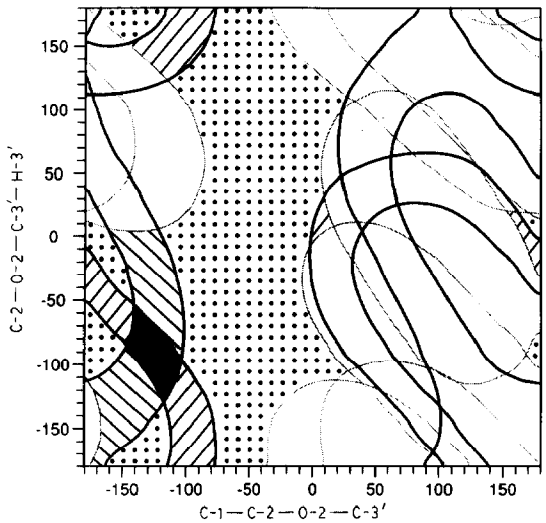

Fig. 5. Overlaid torsion angle maps from Figs. 3b and 4b. The dark area shows the region consistent with all ROE data, the unshaded areas give the regions in which no significant populated conformers can lie.

the negative and positive ROE constraints. Because of the nature of the calculated average for NOEs or ROEs (a $1/r^6$ or square of $1/r^3$ average, not the inverse sixth power of an r average[3]), conformers in which two protons are close in space will be very heavily weighted in the NMR data. For the negative distance constraints, this implies that all significantly populated conformers must satisfy all the constraints (if any one conformer breaks the constraint, then some form of NOE or ROE would be observed), whereas for the positive distance constraints only the appropriate distance average over all conformers must satisfy each constraint. Any single significantly populated conformer or the average (defined by a simple linear distance average) does not need to satisfy any of the positive distance constraints, whereas it must satisfy all the negative distance constraints. Thus, although the information obtained appears to be less useful, negative information obtained from NOE or ROE data can provide more rigorous conformational constraints than positive information.

The conformation of the Lewis X group has already been determined[10,11] and is conserved in sialyl-Lewis X (results to be published elsewhere). The overall conformation of sialyl-Lewis X can then be obtained by putting together the Lewis X group conformation and the α-NeuAc-(2 → 3)-Gal linkage. By inspection, it is obvious that the single conformer for the α-NeuAc-(2 → 3)-Gal linkage in sialyl-Lewis X consistent with the NMR data is unlikely to be the true solution conformation because of very unfavourable steric interactions between the NeuAc and the Gal. Thus, sialyl-Lewis X must exist in solution as a series of conformers in fast exchange. A previous study of the α-NeuAc-(2 → 3)-Gal linkage in the simple

disaccharide and the G_{M4} ganglioside[12], using energy-minimisation calculations, suggests that there are three populated conformers for the linkage. Of these, two are consistent with the negative NOE constraints presented above ($\phi = -153$, $\psi = -26$ and $\phi = -85$, $\psi = 12$) whilst the third ($\phi = 61$, $\psi = -14$) lies in a disallowed region of conformational space. The NMR studies of these two compounds[12] suggest that the third conformer is populated in the G_{M4} ganglioside but not in the simple disaccharide. This is a contrast to energy-minimisation results presented for sialyl-Lewis X and sialyl-Lewis A[13] where a single conformer for each is suggested.

The accurate calculation of the proportions of the different conformers from the ROE data would be very complex, involving calculation of the Hartmann–Hahn exchange contribution[4] to the ROE (rather than an estimate of errors as is used in the qualitiative approach above). An accurate analysis would also require a molecular dynamics simulation of the linkage, as the widths of the potential energy wells and the way that the oligosaccharide explores the available conformational space are required as well as the positions of the absolute minima (as given above) for correct averaging of the ROE. It is worth noting that information on the proportions of different conformers can only be obtained from the positive distance constraints, the negative distance constraints giving no information about the population of any conformer (except for a conformer with zero population).

The interpretation of negative information from NOE or ROE experiments has to be done with some caution. However, in the study of glycosidic linkage conformations, the presence of one cross-linkage NOE or ROE allows safe use to be made of the absence of other cross-linkage NOEs or ROEs. The systematic inclusion of such negative NOE or ROE constraints then provides considerable extra conformational information.

NMR conformational constraints are also used in combination with various theoretical approaches to the determination of oligosaccharide conformations[14,15]. The extra conformational constraints provided by the absence of NOEs or ROEs will enable a more rigorous test of the theoretical results, and will also provide constraints against which each thermally populated conformer, rather than just the average, can be tested.

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