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Sequence Determination for *N*-linked Oligosaccharides through the Use of the Nuclear Overhauser Effect*

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A nuclear magnetic resonance technique, the nuclear Overhauser effect, has been used to confirm the presence of Manα1-2, GlcNAcβ1-2, and Manα1-3 residues and to establish the branching pattern and sequences in four classes of *N*-linked oligosaccharides. This method offers a rapid and nondestructive approach to the elucidation of sequences in carbohydrate chains, in contrast to enzymatic and chemical methods.

High resolution NMR is now a well established method for the determination of the primary structure of asparagine-linked oligosaccharides (1, 2). Its success is based on the fact that the chemical shifts of the H1 and H2 of mannose residues and of the H1 of other hexoses can be correlated with specific sequences and branching patterns obtained from studies of model compounds (1). However, due to the complexity of the spectrum and the similarity of the various components in the branches of glycopeptides, the determination of the sequence in those branches is often difficult and one must resort to enzymatic degradation, methylation analysis, or acetolysis to obtain an unequivocal assignment (3). Since the latter methods are time consuming and destructive, we present in this paper a rapid, nondestructive approach which can be used to confirm the sequences deduced solely from ^{1}H NMR chemical shift data. This method, based on the nuclear Overhauser effect, is applied to four types of *N*-linked oligosaccharides.

The nuclear Overhauser effect has recently been applied to the determination of sequences in glycosphingolipids (4, 5) and to the assessment of oligosaccharide solution conformations (6–8).

EXPERIMENTAL PROCEDURES

All glycopeptides were generous gifts from Dr. H. Schachter (Hospital for Sick Children, Toronto) (9, 10). Samples were prepared in 99.96% D_2O, at concentrations of 10–30 mM, as previously described (1). All experiments were performed on a Nicolet 360 MHz spectrometer located at the Toronto Biomedical NMR Center. All spectra were recorded at 23 ± 2 °C and chemical shifts were calculated relative to internal acetone set at 2.225 ppm. All NOE[1] spectra were obtained by difference spectroscopy (11). Usually, 512 difference free induction decays of 8K points, using quadrature phase detection, were accumulated and Fourier transformed with an exponential line broadening factor of 1.0 in order to improve the signal to noise ratio.

The residual HDO resonance was always a problem because it occurred at 4.8 ppm at 23 °C and overlapped with signals from the oligosaccharide. In addition, the signal intensity was usually too large compared to the glycopeptide signals to get an adequate signal to noise ratio in the NOE spectra. In order to reduce the intensity of the HDO signal, it was selectively inverted by a long soft pulse before each accumulation and the acquisition was initiated when the HDO signal was at a null.

RESULTS AND DISCUSSION

The nuclear Overhauser effect is defined as the fractional change in the integrated intensity of a signal brought about by the saturation of another signal in the spectrum under well defined experimental conditions. Of the many factors which influence the magnitude of this effect, the most important is the r^{-6} dependence of the NOE on a proton resonance, where r is the distance between this proton and the proton whose signal has been saturated. The sign and magnitude of the NOE also depends on the relative orientation of neighboring protons ("three spin effects"), on the Larmor frequency (ω_0) of the hydrogen nuclei and on the tumbling time (τ_c) of the molecules (12, 13). Since the products of the latter two factors were similar for all the present compounds (*i.e.* $\omega_0\tau_c \sim 2$ to 2.5), negative NOEs of similar magnitude were observed in all cases. As an example, when saturating the H1 resonance of an α-D-mannopyranoside residue at 5.115 ppm in the glycopeptide GGN (Fig. 2*c*, see Fig. 1 for nomenclature), a negative NOE of 11% is observed on the H2 signal of the same residue at 4.118 ppm. In α-D-mannopyranose, the H2-H1 distance, which is known from crystallographic data, is approximately 2.6 A (14). NOEs on other protons ($r > 3.5$ A) of this residue are less than 5%.

In asparagine-linked oligosaccharides, one can use this technique to sequence sugars because of the nature of their structure and their ^{1}H NMR spectra. The different classes of *N*-linked carbohydrates are classified according to the pattern of substitution of their core mannotrioside (1, 2). Depending on the pattern of substitution, the H1 and H2 resonances of the mannoses along with the anomeric resonances of other residues have characteristic chemical shifts which occur in an isolated region of the spectrum from 5.4 to 4.0 ppm. For the structures considered here, these resonances have all been assigned previously (2, 3, 9). The H1 and H2 signals of mannose residues are particulary amenable to NOE experiments since their multiplet patterns are narrow. Thus, less power is required to saturate these resonances and better selectivity can be achieved.

In general, when saturating the resonance of a certain proton of a residue (the glycon), NOEs can be observed on proton resonances of the aglycon as well as on the glycon. Sometimes, depending on the linkage, enhancements are also observed on proton resonances of residues linked to the glycon. Valuable sequence information can be obtained from these inter-residue NOEs. For α1-2, β1-2, and α1-3 linkages to mannose, inter-residue NOEs are observed in the 5.4 to 4.0 ppm region. Since all the resonances have been assigned in that region of the spectrum, one can easily deduce which residues are linked together.

Only a qualitative analysis of the NOEs in the 5.4 to 4.0

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[1] The abbreviation used is: NOE, nuclear Overhauser enhancement.

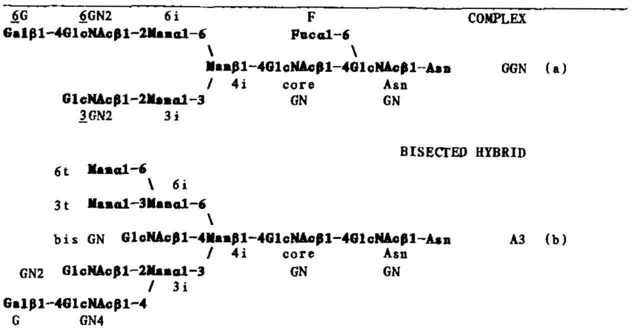

FIG. 1. **Nomenclature for Asn-linked oligosaccharides.** Only a complex structure (*a*) and a bisected hybrid structure (*b*) are shown, since the other structures presented in this paper can be derived from these. For complex structures, to indicate whether a residue is located on the 3-arm or the 6-arm of the core mannotriose unit, an *italicized number* (*3* or *6*) precedes the name of the residue.

ppm region of the spectrum will be presented here, since this is the only information necessary to deduce the sequence of residues. The quantitative analysis, which has been used to determine the solution conformation of *N*-linked oligosaccharides will be presented elsewhere.[2]

In a biantennary complex structure, the mannotriose unit is substituted by Galβ1-4GlcNAcβ1- arms (Fig. 1*a*). Units linked to the Manα1-6 residue of the mannotrioside are said to be located on the 6-arm, while those units linked to the Manα1-3 of the core mannotrioside are located on the 3-arm. The unequivocal assignment of the resonances arising from the residues in those arms is difficult because the branches have similar composition. In GGN, the chemical shift of the H1 resonances of the GlcNAc residues are different because only one is substituted by Gal. Since the H1 and H2 resonances of the Man residues are not appreciably affected by the presence of a terminal Gal residue in the structure (9), it is difficult from chemical shift data alone to tell on which arm the Gal residue is situated. However, the location of the Gal residue in GGN and the unequivocal assignment of the GlcNAc and Gal H1 resonances can be deduced from NOE experiments. Saturation of the H1 resonance of the 3i residue (see Fig. 1*a* for nomenclature) yields enhancements on the following signals: 3i H2, 4i H2, 4i H1, and GN2 H1 (4.553 ppm) (Fig. 2*c*). Since the intensity of the latter is similar to that of the 3i H2 NOE (~10%), the GN2 H1 must be within 2.6 A of the 3i H1. The close proximity between these two atoms can only arise if these two residues are linked. The NOEs on the 4i H2 and H1 signals are a consequence of the α1-3 linkage between the two Man residues in the GGN structure. Although 4i H1 and H2 are more than 4 Å away from 3i H1, small negative NOEs of 2 and 4%, respectively, are observed as a consequence of the three spin effect (13). The 4i H1 and H2 relax through mutual dipolar interactions with the 4i H3 which exhibits a large negative NOE of ~20% (not shown) because of its proximity to the 3i H1. Hence, this very specific chain of NOEs on the 4i H3, H2, and H1 resonances is highly useful in establishing the sequence Manα1-3Manβ1-. The above enhancements thus confirm the sequence GlcNAcβ1-2Manα1-3Manβ1- and permit the assignment of the 4.553 ppm resonance to H1 of *3*GN2 and the 4.578 ppm resonance to the H1 of *6*GN2 (Fig. 2*b*). By comparing the spectra of GG and GGN (Fig. 2, *a* and *b*), one can deduce that a substitution at C4 on GN2 shifts its H1 resonance downfield by 0.025 ppm (9). Since the two equivalent GN2 H1 resonances of GG have a similar chemical shift with that of the *6*GN2 H1 resonance in GGN, the Gal residue in GGN must be located on the 6-arm and the Gal resonance at 4.471 ppm in GGN can be assigned as *6*G4 H1.

In GG, although the Gal H1 resonances have slightly different chemical shifts, their assignment cannot be determined *a priori*. However, the lowfield component of the G4 H1 resonances in GG can be tentatively assigned as *6*G4 H1 since it has the same chemical shift as the *6*G4 H1 resonance in GGN (4.471 ppm). The highfield component of the Gal H1 resonances in GG (4.466 ppm) then corresponds to *3*G4 H1.

These assignments are identical to those reported for a similar compound with no Fucα1-6 present in the core (2). The latter were deduced from the corresponding oligosaccharide terminating as Manβ1-4GlcNAc(α,β) and from compounds in which the Gal residues were substituted by sialic acid. Thus, the NOE technique permits an alternative way of assigning resonances when appropriately modified compounds are not available.

For the bisected biantennary hybrid C3B, saturation of the 3i H2 resonance (Fig. 3*b*) and saturation of the 3i H1 signal (Fig. 3*c*) can be used to establish the sequence for this compound. Saturation of the H2 resonance at 4.255 ppm yields NOEs on the H1 signal (5.055 ppm) and the GN2 H1 resonance. Upon saturation of the H1 signal at 5.055 ppm, the same NOE pattern as in GGN was observed (inter-residue NOEs on GN2 H1 and 4i H2). The ratio of the enhancements on the 4i H2 to 3i H2 resonances is the same as that found for GGN (Fig. 2*c versus* Fig. 3*c*). If the GN2 residue was linked to the Manα1-3 residue on the 6-arm, one would expect to see an enhancement on the 6i H2 resonance which is comparable to the NOE on the 3i H2 signal. Since this is not the case, the GN2 residue must be located on the 3-arm of the core, thus establishing the sequence GlcNAcβ1-2Manα1-3Manβ1-. The small NOE on the 3t H2 resonance (4.061 ppm, Fig. 3*c*) is caused by the partial saturation (50%) of the 3t H1 resonance (see Fig. 1*b* for nomenclature).

It should be noted that the H1 resonances for the 3i and 3t residues, were interchanged in the original work (3). These assignments were based on decoupling experiments where

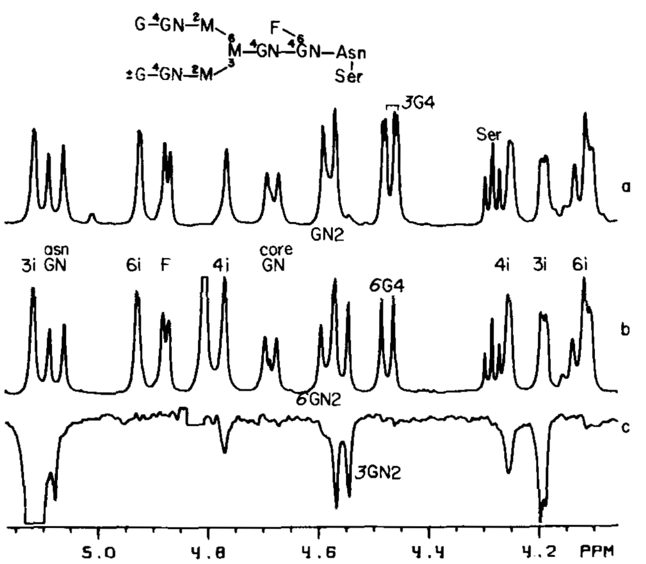

FIG. 2. **Sequence determination for the biantennary complex glycopeptides GG and GGN.** *a*, Spectrum of GG. The residual HDO signal at 4.8 ppm has been saturated. *b*, Spectrum of GGN. *c*, NOE difference spectrum of GGN: saturation of the 3i H1 resonance at 5.115 ppm. The HDO signal (4.8 ppm) has been removed. In all these figures, the biggest NOE, which is about 10% of the saturated resonance, has been set to full scale.

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[2] Brisson, J.-R., and Carver, J. P., manuscript in preparation.

changes in signal intensities brought about by the loss of a coupling between protons are often difficult to detect when the coupling constants involved are of the same order as the line widths of the resonances (1-2 Hz). This is usually the case for the H1 resonances of mannose residues in these high molecular weight compounds. Thus, NOE experiments, which are not affected by these restraints, can be extremely useful in establishing assignments in such situations.

As can be observed in the NMR spectrum of the bisected triantennary hybrid A3, the only isolated resonances are the 4i H1 and the 3i H2 (Fig. 4*a*). Saturation of the former (Fig. 4*b*) confirms the sequence Manα1-3Manβ1-, since small inter-residue enhancements are observed on the 3i H1 and H2 signals (5.048 and 4.290 ppm, respectively). As explained above, these NOEs are a result of a three spin effect between 4i H1, 4i H2, and 3i H1 which arises because they are adjacent in space and relax mutually with each other (13). There is no significant relaxation pathway between 6i H1 and 4i H1 which would allow, upon saturation of the 4i H1 resonance, NOEs to occur on the H1 and H2 signals of the Manα1-3 residue on the 6-arm. The small intensity on the core GN H1 is probably due to partial saturation, while the signals near 4.88 ppm probably arise from a spinning sideband and partial saturation of the 6i H1 resonance. The small NOE on the bis GN H1 (4.413 ppm) is probably a consequence of mutual dipolar interactions between protons in the GlcNAcβ1-4Manβ1- unit.

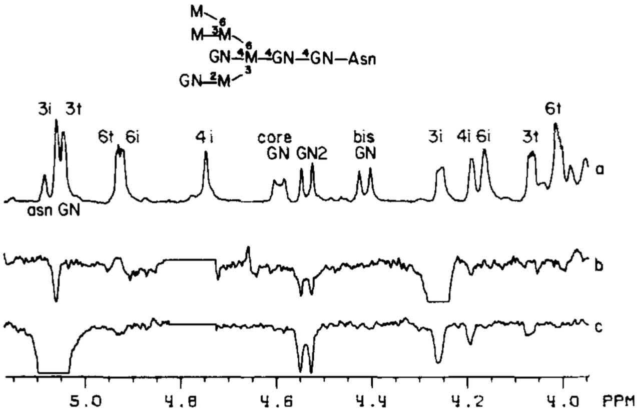

FIG. 3. **Sequence determination for the bisected biantennary hybrid glycopeptide C3B.** *a*, Spectrum of C3B. The broad line shape of the core GN H1 resonance is due to virtual coupling (15). The HDO signal (4.8 ppm) has been saturated. NOE difference spectra: *b*, saturation of the 3i H2 resonance at 4.255 ppm; *c*, saturation of the 3i H1 resonance at 5.055 ppm. The 3t H1 signal is also partially saturated (50%). The HDO signal (4.8 ppm) has been removed.

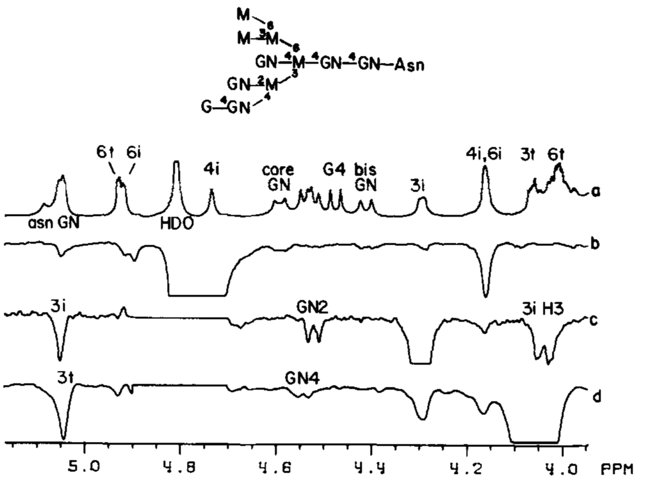

FIG. 4. **Sequence determination for the bisected triantennary hybrid glycopeptide A3.** *a*, Spectrum of A3. The line shape of core GN H1 resonance is also affected by virtual coupling (see Fig. 3). NOE difference spectra: *b*, saturation of the 4i H1 resonance at 4.730 ppm; *c*, saturation of the 3i H2 resonance at 4.290 ppm, *d*, saturation of the 3i H3, 3t H2, 6t H2 resonances around 4.02 ppm. In *b* and *c*, the large HDO signal (4.8 ppm), which in *b* probably obscured a small NOE (1%) on 4i H1, has been removed.

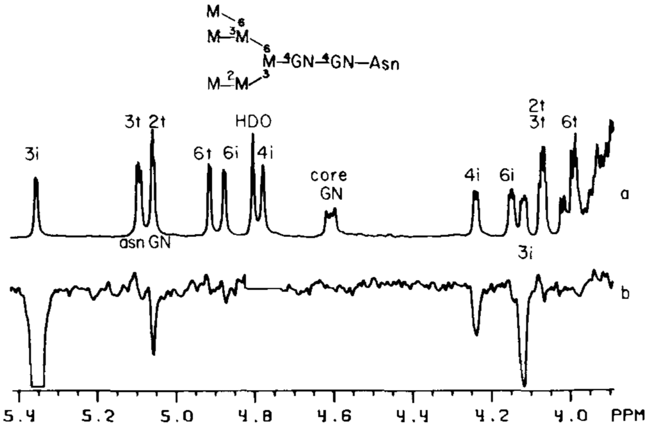

FIG. 5. **Sequence determination for the high mannose glycopeptide D3.** *a*, Spectrum of D3, the line shape of the core GN H1 signal is also due to virtual coupling (see Fig. 3). *b*, NOE difference spectrum with saturation of the 3i H1 signal at 5.348 ppm. The large HDO signal (4.8 ppm), which probably obscured a small NOE on the 4i H1 resonance, has been removed.

TABLE I

Nuclear Overhauser enhancements that can be used to confirm some sequences in Asn-linked oligosaccharides

The saturated signal is denoted by *. An observable NOE is denoted by + and the absence of an NOE is denoted by −. *a* indicates a signal that is partially saturated due to its proximity to the saturated signal and *b* indicates a probable NOE which is obscured by the large HDO signal.

Sequence	Residues					
	A Manβ1-		B Manα1-3		C Manα1-2 or GlcNAcβ1-2	
C B A	H1	H2	H1	H2	H1	H2
Manα1-2Manα1-3Manβ1-	*	+	+	−		
	+	*	+	*a*		
	−	+	*	+	+	−
	b	*a*	+	*	+	*a*
	−	−	+	+	*	+
	−	−	−	−	+	*
GlcNAcβ1-2Manα1-3Manβ1-	*b*	+	*	+		
			+	*	+	
	−	−	+	+	*	

As in C3B, saturation of the 3i H2 resonance in A3 (Fig. 4*c*) confirms the sequence GlcNAcβ1-2Man. However, a new NOE appears at 4.037 ppm. From its coupling constants and intensity, it can be easily assigned as 3i H3. In C3B, this proton resonates at 3.88 ppm.[2] This shift of 0.16 ppm is characteristic of a substitution at C4 (15). Hence, the 3i residue must be doubly substituted at its C2 and C4 positions by GlcNAc. Further evidence supporting the C4-substitution is obtained upon saturation of the resonances (3i H3, 3t H2, 6t H2) near 4.03 ppm (Fig. 4*d*), since a small NOE (1%) is observed on the GN4 H1 at 4.535 ppm. This NOE can only arise through mutual dipolar relaxation with protons involved in the GlcNAcβ1-4Manα1- unit located on the 3-arm, since the 3t and 6t residues are too far removed from GN4. In Fig. 4*d*, the other enhancements (3t H1, 6t H1, and 3i H2) are all intra-residue NOEs.

For the high mannose compound D3, the position of the Manα1-2 residue (2t) in the structure cannot be readily determined solely from chemical shift data (3). However, upon saturation of the 3i H1 resonance (Fig. 5*b*) NOEs are detected on the 3i H2, on the 4i H2, and on the 2t H1 signals, thus establishing the sequence Manα1-2Manα1-3Manβ1-. Once again, the NOE on the 4i H2 arises from a three spin effect.

Thus, NOEs can be used to establish sequences that contain Manα1-2Man, Manα1-3Man, and GlcNAcβ1-2Man units. The H1 resonances of hexoses and the H2 resonances of mannoses can be saturated to obtain sequencing information. The variations that are possible for the above linkages are listed in Table I. The availability of several approaches permits considerable flexibility in choosing suitably isolated resonances for the NOE experiment. Also, since this technique permits the observation of only those protons that are in close proximity to the saturated proton, its use as a tool for assigning new resonances should be quite general.

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