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The potency of amide protons for assignments of NMR spectra of carbohydrate chains of glycoproteins, recorded in ^1H_2O solutions

Karl Hård, Bertina A. Spronk, Cornelis H. Hokke, Johannis P. Kamerling and Johannes F.G. Vliegenthart

Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80075, NL-3508 TB Utrecht, The Netherlands

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Three glycoprotein *N*-glycans, namely, a disialylated diantennary carbohydrate chain linked to Asn, a monosialylated, fucosylated diantennary glycopeptide with bisecting *N*-acetylglucosamine, and a tetrasialylated, fucosylated tetra-antennary oligosaccharide, have been investigated by two-dimensional NOE and HOHAHA spectroscopy in ^1H_2O as solvent. The amide protons of all *N*-acetylglucosamine and sialic acid residues could readily be assigned. The large chemical-shift dispersion of the amide resonances of the *N*-acetylglucosamine residues, allowed the unambiguous assignment of every *N*-acetyl methyl signal, via strong NOEs. Subspectra could be obtained of all *N*-acetylglucosamine residues in HOHAHA spectra. These results have as main implication that several biologically important large glycans, will not become amenable for conformational studies by multidimensional NMR in ^1H_2O solution.

Carbohydrate, *N*-linked; 2D NMR

1. INTRODUCTION

^{1}H-NMR spectroscopy is widely used to determine the structure of complex carbohydrates [1–6]. A general feature of ^{1}H-NMR spectra of carbohydrates is the small chemical-shift dispersion of the protons, since the majority of the resonances are found in a crowded region between 3.5 and 4.0 ppm. A few protons, the so-called structural-reporter groups, resonate outside this 'bulk'-region, and they can be used to gain structural information [1,6]. Sofar, many of the spectral assignments, like those of *N*-acetyl methyl signals, are based on comparisons of a vast number of structurally related compounds. ^{1}H-NMR studies on oligosaccharides and glycopeptides have usually been performed in deuterium oxide (^2H_2O). Owing to exchange, hydroxyl and amide protons are generally not observed in ^2H_2O.

In the present report results are shown from 2D ^{1}H-NMR studies in ^1H_2O, instead of ^2H_2O, of three sialylated N-linked carbohydrate chains. By taking advantage of the favorable chemical-shift dispersion of the amide resonances several unambiguous assignments, unattainable in deuterium oxide, have been made.

Correspondence address: J.F.G. Vliegenthart, Bijvoet Center, Department of Bio-Organic Chemistry, Utrecht University, P.O. Box 80.075, NL-3508 TB Utrecht, The Netherlands. Fax: (31) (30) 540980.

Abbreviations: Fuc, fucose; HOHAHA, homonuclear Hartmann–Hahn; MLEV, M. Levitt; Neu5Ac, *N*-acetylneuraminic acid; NOE, nuclear Overhauser enhancement; NOESY, 2D NOE spectroscopy; TPPI, time-proportional phase increment; 2D, two-dimensional.

2. MATERIALS AND METHODS

2.1. *Materials*

The structures of the compounds used in this study are depicted in Scheme 1. Compound **1** (ca. 20 mg) was a gift of Profs. J. Montreuil and G. Spik (Université des Sciences et Techniques de Lille Flandres-Artois, France), compound **2** (ca. 5 mg) was obtained by protease digestion of human plasma IgM stemming from a patient suffering from Waldenström's macroglobulinemia, and compound **3** (ca. 1 mg) was obtained by enzymatic digestion with peptide-N^4-(*N*-acetyl-β-glucosaminyl)asparagine amidase F (PNGase-F) of recombinant human erythropoietin [7]. Deuterium oxide (99.9 atom-% ^{2}H) was purchased from MSD Isotopes.

2.2. *^{1}H-NMR spectroscopy*

The samples were dissolved in 450 μl 20 mM potassium phosphate, containing 90% (v/v) ^1H_2O, 10% (v/v) ^2H_2O, and 0.02% (w/v) sodium azide. The pH was adjusted to 5.2 by addition of dilute HCl or NaOH. ^{1}H-NMR spectra were recorded at 27°C on a Bruker AM-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) or on an AM-600 spectrometer (SON hf-NMR facility, Department of Biophysical Chemistry, University of Nijmegen). The time-proportional phase increment method (TPPI) [8] was used for phase-sensitive two-dimensional (2D) NOE [9] and 2D HOHAHA [10] experiments. The 2D NOE spectra were recorded with a 200-ms mixing time. The 2D HOHAHA spectra were recorded with a 100–120-ms spin-locking time using a MLEV-17 pulse sequence, sandwiched between two 2-ms trim-pulses. The 90° ^{1}H-pulse, in the MLEV-17 pulse sequence, was adjusted to 25–30 μs. The carrier frequency was placed on the water resonance, which was presaturated for 1 s during the relaxation period. Two-dimensional spectra were recorded with 400–512 t_1 increments, and 19–96 free induction decays of 2048 data points per t_1 value were collected. All data processing was carried out on a VAXstation 3100 using the TRITON NMR software (Bijvoet Center, Department of NMR Spec-

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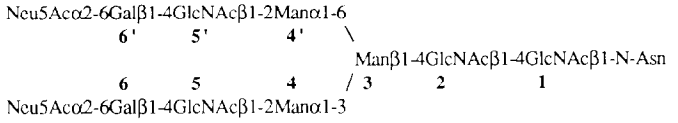

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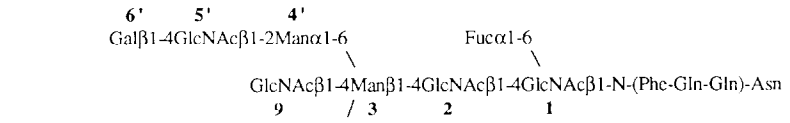

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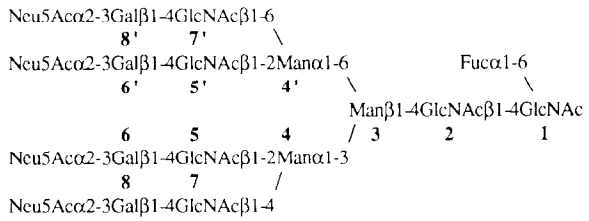

3

Scheme 1

troscopy, Utrecht University). The time-domain data were multiplied with a phase shifted sine-bell. After Fourier transformation the resulting data set of 1024 × 1024 points was baseline-corrected in both frequency domains by a fourth order polynomal fit [11]. Chemical shifts are given by reference to internal acetone (δ 2.225) [1].

3. RESULTS

Preliminary ^{1}H-NMR experiments in ^1H_2O, using *N*-acetylglucosamine and *N,N'*-diacetylchitobiose as model compounds, indicated that at pH >7 the amide proton signals in the ^{1}H-NMR spectra become very weak (data not shown). The intensities of the amide proton resonances are only slightly temperature-dependent in the region 9–39°C (data not shown). At 27°C and pH 5.2, the amide proton resonances of compounds **1–3** are readily observed. In Table I relevant ^{1}H-NMR assignments for **1–3** are presented. Parts of the 2D NOE and 2D HOHAHA spectra of **1–3** in ^1H_2O are shown in Fig. 1. The strategy for carrying out the assignments of the amide, *N*-acetyl methyl, and anomeric proton resonances of **1–3** will be discussed in detail, using **1** as an example.

For the disialylated diantennary glyco-amino-acid **1**, the Asn residue is recognized from its unique spin system in the HOHAHA spectrum (Hα, δ 4.53; Hβ's, δ 2.73 and 2.81; αNH_2, δ 8.34) (Fig.1D). The side-chain amide proton (γNH, δ 8.53) of Asn is identified from NOEs to the β-protons of that residue, from a NOE to the H-2 of GlcNAc-**1** (δ 3.85) [12] (Fig. 1A), as well as from HOHAHA connectivities to GlcNAc-**1** NH (δ 8.21), H-1 (δ 5.050), H-2 (δ 3.85), and H-3 (δ 3.76). In general, once an amide proton signal of a *N*-acetylglucosamine or *N*-acetylneuraminic acid residue has been located, the corresponding *N*-acetyl methyl signal can be found from a strong NH→CH_3 NOE. In a GlcNAcβ1-2Manα element the NOE between GlcNAc NH and Man H-1 can be used to define its branch location. For example, GlcNAc-**5** and GlcNAc-**5'** NH resonances (δ 8.21 and 8.19, respectively) can be distinguished by use of their NOEs to the Man-**4** (δ 5.135) and Man-**4'** (δ 4.945) H-1 signals, respectively. Previously, the Man H-1 atoms, which belong to the 'classical' structural-reporter groups, have been assigned [1], and proven by 2D [13], and 3D NMR studies [12]. The resonances of the amide protons of both Neu5Ac residues coincide at δ 8.02. Their positions were located by their characteristic HOHAHA connectivities to the Neu5Ac H-3a (δ 1.71) and H-3e (δ 2.67) signals. Finally, a NOE from the GlcNAc-**2** CH_3 signal (δ 2.079) affords the chemical shift (δ 8.41) of the NH atom of this residue.

The amide, *N*-acetyl methyl, and anomeric proton resonances of the monosialylated, fucosylated, bisecting-GlcNAc-containing glycopeptide **2** can be assigned in an analogous way as described for **1** (see Table I, and Fig. 1B,E). The amide proton signal of bisecting GlcNAc-**9** (δ 8.37) is found from its HOHAHA connectivities to the well-resolved H-4 (δ 3.273) and H-5 (δ

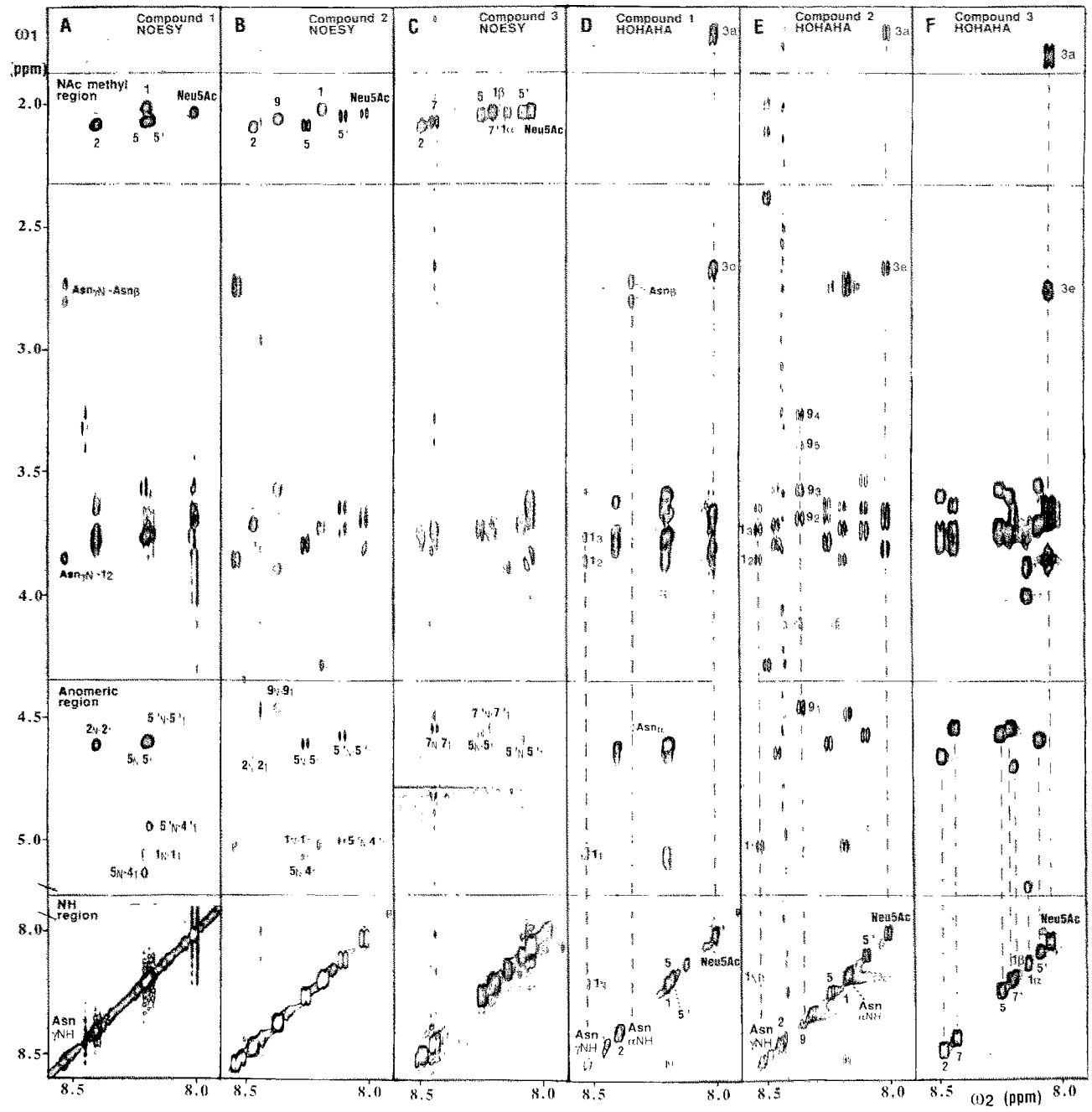

Fig. 1. Parts of the NOESY spectra (A, compound **1**; B, compound **2**; C, compound **3**) and 2D HOHAHA spectra (D, compound **1**; E, compound **2**; F, compound **3**) recorded at 27°C in $^1H_2O/^2H_2O$ (9/1, v/v), pH 5.2, containing 20 mM potassium phosphate. The spectrometer frequency was 600 MHz in A and 500 MHz in all other cases. In the 'NAc methyl region' of the NOESY spectra numbers at intraresidual (NH→NAc CH_3) cross-peaks refer to the corresponding sugar residue number (see Scheme 1). In the 'anomeric region' of the NOESY spectra, $\mathbf{5}_N$–$\mathbf{4}_1$ indicate a cross-peak between the amide proton of GlcNAc-**5** and Man-**4** H-1, and so on. The positions of all amide resonances are given on the diagonals in the 'NH region' of the HOHAHA spectra, and numbers indicate sugar residue numbers (see Scheme 1). In the HOHAHA spectra, cross-peaks on the amide tracks are labeled as follows: $\mathbf{Asn}_\alpha$, Asn H-α; $\mathbf{Asn}_\beta$, Asn H-β; 3a, Neu5Ac H-3a; 3e, Neu5Ac H-3e; $\mathbf{1}_N$, GlcNAc-1 NH; $\mathbf{1}_1$, GlcNAc-1 H-1; $\mathbf{1}_2$, GlcNAc-1 H-2; etc.

3.404) atoms of this residue. It should be noted that the NH resonance of GlcNAc-**2** has shifted downfield ($\Delta\delta$ 0.06) when compared to compound **1**. This shift is most likely an effect of the presence of the α1-6-linked Fuc at GlcNAc-**1**.

Compound **3** is a reducing tetrasialylated, fucosylated tetra-antennary carbohydrate chain. Extensive overlap of the anomeric signals of Gal-**6**, Gal-**6'**, GlcNAc-**7**, GlcNAc-**7'**, and Gal-**8**, prevents unambiguous assignment of these signals, when the spectrum

is recorded in ^2H_2O (Table I). However, by recording a 2D HOHAHA spectrum in ^1H_2O, subspectra of all *N*-acetylglucosamine residues can be obtained by starting from the amide protons (Fig. 1F). The position of the GlcNAc-**1**α NH resonance was deduced by locating the scalar NH→H-1 (δ 8.15→δ 5.182) connectivity in the HOHAHA spectrum. The well-resolved H-1 signal of GlcNAc-**1**α is easily assigned from spectra recorded in ^2H_2O [14]. The GlcNAc-**1**β NH signal (δ 8.20) is inferred from its HOHAHA connectivity to GlcNAc-**1**β H-1 (δ 4.688). Correspondingly, the GlcNAc-**2** NH resonance (δ 8.48) is deduced from its HOHAHA cross-peak to GlcNAc-**2** H-1 (δ 4.66). The positions of the NH signals of GlcNAc-**5** (δ 8.25) and GlcNAc-**5′** (δ 8.09) were also judged from HOHAHA connectivities to their corresponding H-1 atoms at δ 4.563 and 4.593, respectively. The chemical shifts of the GlcNAc-**5** and GlcNAc-**5′** H-1 signals have previously been proven by NOEs with the H-1 resonances of Man-**4** and Man-**4′**, respectively [15]. The GlcNAc-**7** and GlcNAc-**7′** amide proton resonances (δ 8.43 and 8.20, respectively) were discriminated via NOEs to the well-separated CH_3 protons of these residues (Fig. 1C), previously assigned on basis of suitable model compounds. Subsequently, the H-1 signals of GlcNAc-**7** and GlcNAc-**7′**, partially overlapping with Gal-**6**, Gal-**6′**, and Gal-**8**, are assigned using NOEs to the corresponding NH atoms. The amide proton resonances of the four terminal α2-3-linked Neu5Ac residues all coincide at δ 8.05, whereas the amide proton signals of the α2-6-linked sialic acids in compounds **1** and **2** resonate at δ 8.02 (see Table I).

Table I

^1H chemical shifts of H-1, CH_3, and NH atoms of constituent monosaccharides in compounds **1**, **2** and **3**. Chemical shifts are given at 27°C (except the H-1 and CH_3 values of **1** at 31°C) by reference to internal acetone (δ 2.225) [1]. The chemical shifts for the H-1 and CH_3 atoms were obtained from 1D spectra in ^2H_2O, and those for the NH atoms for 2D NOE and 2D HOHAHA spectra in $^1H_2O/^2H_2O$ (9/1, v/v) containing 20 mM potassium phosphate, pH 5.2. For numbering of the monosaccharide residues, see Scheme 1

Compound	Residue	Reporter group		
		H-1	CH_3	NH
		(ppm)		
1	GlcNAc-**1**	5.050	2.012/2.008	8.21
	GlcNAc-**2**	4.62	2.079	8.41
	GlcNAc-**5**	4.605	2.068	8.21
	GlcNAc-**5′**	4.605	2.065	8.19
	Gal-**6**	4.443	–	–
	Gal-**6′**	4.447	–	–
	Neu5Ac	–	2.030[a]	8.02[a]
2	GlcNAc-**1**	5.018	2.007	8.20
	GlcNAc-**2**	4.657	2.091	8.47
	GlcNAc-**5**	4.616	2.080	8.26
	GlcNAc-**5′**	4.576	2.040	8.11
	Gal-**6**	4.444	–	–
	Gal-**6′**	4.475	–	–
	GlcNAc-**9**	4.467	2.055	8.37
	Neu5Ac	–	2.030	8.02
3	GlcNAc-**1**α	5.182	2.038	8.15
	β	4.688	2.038	8.20
	GlcNac-**2**α[b]	4.659	2.094	8.48
	β	4.664	2.090	8.48
	GlcNAc-**5**	4.563	2.047	8.25
	GlcNAc-**5′**	4.593	2.038	8.09
	Gal-**6**	4.542[c]	–	–
	Gal-**6′**	4.545[c]	–	–
	GlcNAc-**7**	4.542	2.075	8.43
	GlcNAc-**7′**	4.545	2.038	8.20
	Gal-**8**	4.542[c]	–	–
	Gal-**8′**	4.559	–	–
	Neu5Ac	–	2.031[d]	8.05[d]

[a] Signal belonging to two Neu5Ac residues
[b] α and β refer to the anomeric configuration of GlcNAc-**1**
[c] Assignments may have to be interchanged
[d] Signal belonging to four Neu5Ac residues

4. DISCUSSION

In the present report it is shown that by measuring ^1H-NMR spectra in ^1H_2O, it is possible to relate a monosaccharide *N*-acetyl methyl group via a NOE to the corresponding amide proton. By HOHAHA cross-peaks the amide proton resonances of GlcNAc and Neu5Ac can be connected to protons belonging to the respective monosaccharide ring spin system. Several assignments, that have previously been made by extensive comparisons of closely related compounds, have now been proven unambiguously. Furthermore, the amide resonances of *N*-acetylglucosamines have a favorable chemical-shift dispersion. Subspectra of all *N*-acetylglucosamine residues can be obtained, even for an α2-3-tetrasialylated tetra-antennary *N*-acetyllactosamine type carbohydrate chain.

Carbohydrate amide proton resonances have previously been observed by ^1H-NMR spectroscopy in aqueous solution [16–18], but no advantage has been taken of the excellent chemical-shift dispersion of these protons for specific assignments. ^1H-NMR spectroscopic observation of fast exchanging hydroxyl protons demands a lower temperature and softer water suppression scheme, than was used in the present investigation [18,19]. Moreover, a drawback of the use of hydroxyl protons is the huge amount of hydroxyl groups in larger oligosaccharides. Therefore, it is expected that the use of amide protons will provide a more general tool for structural studies on complex carbohydrates. In particular, a combination of multidimensional (heteronuclear) NMR spectroscopy in ^1H_2O solution and computational simulations should enable detailed conformational studies of biologically interesting, large carbohydrate chains.

In protein NMR spectroscopy the use of amide resonances forms a cornerstone for structure determination [20]. Most amide protons in proteins are linked to nitrogens involved in interresidual peptide linkages, making them sensitive to secondary and ter-

tiary structural features. This contrasts with carbohydrate chains of glycoproteins, where all amide protons are part of exocyclic substituents. However, here it could be shown that the chemical shifts of carbohydrate amide protons are very sensitive to subtle structural differences, e.g. the GlcNAc amide protons in the four Neu5Acα2-3Galβ1-4GlcNAcβ1 branches in a tetrasialylated, tetra-antennary carbohydrate chain all resonate at clearly distinct positions.

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