HIGH RESOLUTION ^{1}H-NMR SPECTROSCOPY IN THE STRUCTURE ANALYSIS OF CARBOHYDRATES DERIVED FROM GLYCOPROTEINS

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Abstract – The suitability of 360-MHz ^{1}H-NMR spectroscopy for the unequivocal determination of the carbohydrate composition and primary structure of N- or O-glycosidically linked carbohydrate moieties of glycoproteins is described. The spectra, recorded in deuteriumoxide, show that several protons resonate at well defined positions, clearly separated from the bulk of the skeleton protons. The chemical shifts and coupling constants of the former protons are characteristic for the primary structure of the compounds. This opened the possibility to use these protons as structural reporter groups for the elucidation of carbohydrate structures related to compounds discussed in this paper. The NMR method has the advantage of being non-destructive. Only 100 nmoles of a pure substance are needed for this type of analysis.

Several suggestions on the physiological function and way of action of the carbohydrate chains of glycoproteins have been based on specific carbohydrate structures and observed alterations therein (1-6). Therefore an unambiguous determination of the primary structure of these carbohydrate chains is of the utmost importance. However, these structural studies are hampered by several causes. The first complication is that in general it is difficult to isolate a glycoprotein in the form of a single molecular species. Natural or artificial (micro)heterogeneity of the carbohydrate chains is frequently occurring, giving rise to more or less complex mixtures of closely related compounds. Secondly, intact glycoproteins can not be used so far for the identification of the carbohydrate chains. Degradation to partial structures like glycopeptides, which bear only a few amino acids, oligosaccharides or oligosaccharide-alditols is necessary to make them amenable to structure analysis. Thirdly, the intrinsic limitations of classical methods make it sometimes difficult to arrive at unambiguous results for one and the same glycoprotein, *e.g.* serotransferrin (7,8), α_1-acid glycoprotein (9,10) and fetuin (11,12). This shows that there is an urgent need for methods for check of the purity of compounds used for structural work as well as for the structure determination as such. Besides limitations with regard to the information on the primary structure which can be obtained by classical methods, it should be noted that these techniques are in general highly time consuming. The recent development in NMR instrumentation (13) has enabled the introduction of high resolution ^{1}H-NMR spectroscopy to the determination of the primary structure of complex carbohydrates. As we have shown (10,14-23) for N-glycosidically linked carbohydrate chains of glycoproteins of the lactosamine type, the high resolution ^{1}H-NMR spectra of glycopeptides and oligosaccharides are extremely useful for

a) check of the purity of the compounds
b) determination of the carbohydrate composition by spectral integration
c) elucidation of the primary structure

Furthermore NMR spectroscopy is a rapid and non-destructive method of structure analysis, which leaves open the possibility of subsequent chemical and enzymic studies.

In the last few years we investigated by means of 360-MHz ^{1}H-NMR spectroscopy glycopeptides and oligosaccharides related to various classes of the N-glycosidic structure type. These carbohydrate chains have in common the following structural element:

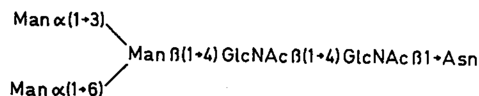

Fig. 1. Pentasaccharide core structure, occurring in glycoproteins with N-glycosidically linked carbohydrate chains (1).

Extension of this core structure with various amounts of N-acetyllactosamine units gives rise to bi-, tri- and tetra-antennary structures, as given in Fig. 2.

```
A
   6        5              4
   Galβ(1→4)GlcNAcβ(1→2)Manα(1→3)\
                                  Manβ(1→4)GlcNAcβ(1→4)GlcNAcβ1→Asn
   Galβ(1→4)GlcNAcβ(1→2)Manα(1→6)/  3          2              1
   6'       5'             4'

B
   8        7
   Galβ(1→4)GlcNAcβ(1→4)
   6        5          \   4
   Galβ(1→4)GlcNAcβ(1→2)Manα(1→3)\
                                  Manβ(1→4)GlcNAcβ(1→4)GlcNAcβ1→Asn
   Galβ(1→4)GlcNAcβ(1→2)Manα(1→6)/  3          2              1
   6'       5'             4'

C
   8        7
   Galβ(1→4)GlcNAcβ(1→4)
   6        5          \   4
   Galβ(1→4)GlcNAcβ(1→2)Manα(1→3)\
                                  Manβ(1→4)GlcNAcβ(1→4)GlcNAcβ1→Asn
   Galβ(1→4)GlcNAcβ(1→2)Manα(1→6)/  3          2              1
   6'       5'         /   4'
   Galβ(1→4)GlcNAcβ(1→6)
   8'       7'
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Fig. 2. Bi- (A), tri- (B) and tetra-antennary (C) structure and the numbering of the sugar residues.

The different branches of these compounds can be terminated by NeuAc, linked α(2→6) or α(2→3) to Gal. Fuc can occur in an α(1→6) linkage to GlcNAc 1 and/or to one or more GlcNAc residues of a lactosamine unit in an α(1→3) linkage. The resonances in a 360-MHz ^{1}H-NMR spectrum of a compound belonging to this type of carbohydrate chains, dissolved in deuteriumoxide, can be divided into signals from the following groups of protons:

i) bulk of the non-anomeric protons giving rise to a broad signal which can not be resolved into contributions of individual protons for relatively large compounds. For small compounds usually a complete interpretation can be achieved.

ii) structural reporter groups which resonate outside of the bulk at well defined, clearly distinguishable chemical shifts and which are in combination with the coupling constants characteristic for the primary structure of the compound. These signals can be subdivided as follows:

a) anomeric protons
b) H-2 protons of Man residues
c) H-3 protons of NeuAc and NeuGl residues
d) H-3 and H-4 protons of Gal residues
e) H-5 and CH_3-group protons of Fuc residues
f) N-acetyl group protons of GlcNAc residues
g) N-acetyl- or N-glycolyl group protons of sialic acid residues

iii) in case of glycopeptides : signals of the amino acid protons.

To illustrate the applicability of the NMR method, we shall discuss in detail the ^{1}H-NMR spectrum of the following generally occurring compound I. The structure and the 360-MHz ^{1}H-NMR spectrum are presented in Fig. 3.

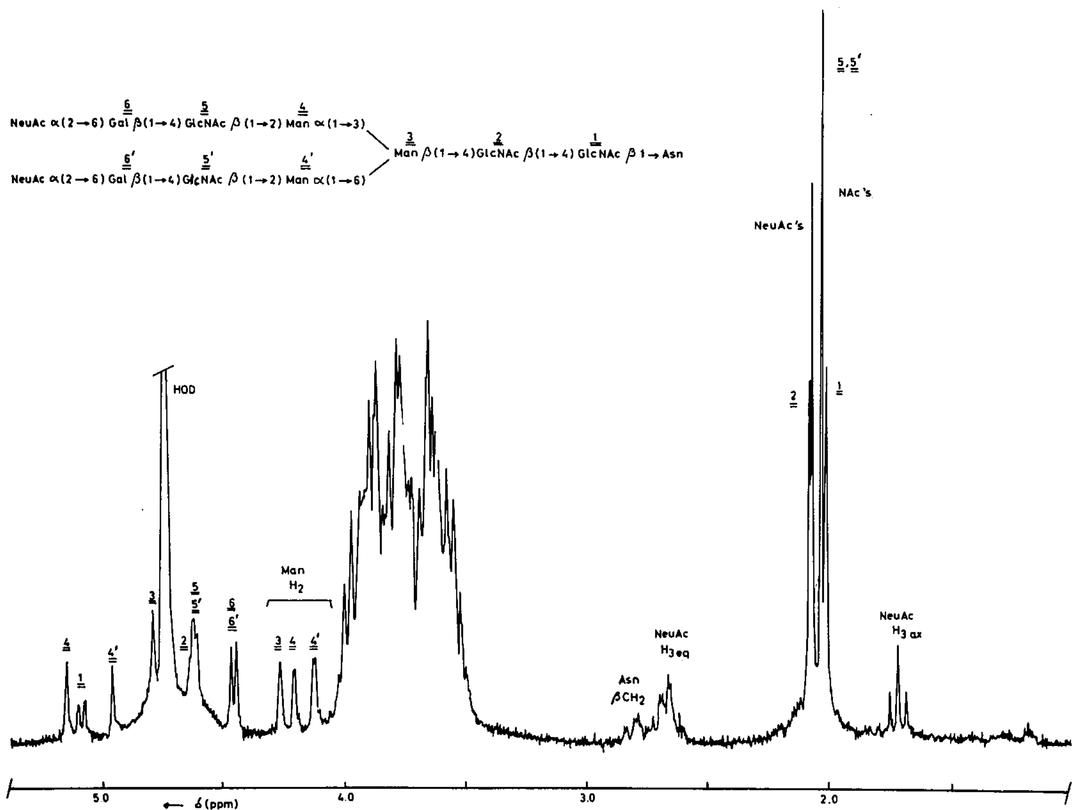

Fig. 3. 360-MHz ^{1}H-NMR spectrum of compound I in deuteriumoxide.

The chemical shifts and coupling constants of the anomeric protons are summarized in Table I.

TABLE I. ^{1}H-NMR chemical shifts of the H-1 protons of compound I and the coupling constants $J_{1,2}$

Residue	Chemical shift (ppm) [a]	Coupling constant $J_{1,2}$ (Hz)
GlcNAc 1	5.075	9.9
GlcNAc 2	4.619	7.5
Man 3	4.772	~ 1
Man 4	5.135	~ 1
Man 4'	4.946	~ 1
GlcNAc 5	4.600	7.5
GlcNAc 5'	4.600	7.5
Gal 6	4.444	7.9
Gal 6'	4.444	7.9

a) Chemical shifts δ are given in ppm downfield from sodium 2,2-dimethyl-2-sila-pentane-5-sulfonate

The anomeric proton of GlcNAc 1 resonates relatively downfield, due to the N-glycosidic linkage. Its value is influenced by the number and type of the amino acids in the peptide portion [δH-1 : 5.05-5.10 ppm (10)]. The β-type of linkage between GlcNAc 1 and Asn can be deduced from the value of the coupling constant $J_{1,2}$ = 9.9 Hz. The chemical environment of the anomeric proton of GlcNAc 2 is greatly different from that of GlcNAc 1, which results in a significantly different chemical shift value. The branching Man residue 3 occupies a specific position in the chain, because it has a β-glycosidic linkage and is substituted by two other Man residues. In accordance with the β-type of linkage the signal of the anomeric proton is found at higher field than those of the other Man residues. This signal is located that close to the HOD-line that it may be hidden under this line at ambient tempera-

ture. A shift of the HOD-line *e.g.* by changing the sample temperature, is then necessary to make it visible. The differences in attachment of Man 4 and 4' to Man 3 are primarily responsible for differences in chemical shifts of their anomeric protons. The α-type of glycosidic linkage can not be distinguished from the β-type on the basis of the coupling constants $J_{1,2}$ due to the equatorial position of H-2 of Man. The resonances from the H-1's of GlcNAc 5 and 5' coincide just like those of Gal 6 and 6'. These GlcNAc and Gal residues have β-glycosidic linkages as can be derived from the coupling constants $J_{1,2}$.
The configuration of the glycosidic linkage of NeuAc can be inferred from the chemical shift of its H-3equatorial proton (24,25). The set of chemical shift values: δH-3axial = 1.715 ppm and δH-3equatorial = 2.671 ppm, is characteristic for a NeuAcα(2→6)Gal sequence (17). Change of the type of linkage to α(2→3) gives rise to the following clearly distinguishable set: δH-3ax = 1.800 ppm, δH-3eq = 2.758 ppm (17). These values are restricted to glycopeptides and oligosaccharides from N-glycosidically linked carbohydrate chains of the lactosamine type [*vide infra*]. Comparison of the chemical shifts of the anomeric protons of compound I with those of the corresponding asialo-compound shows that the presence of α(2→6) linked NeuAc residues has significant effects on the chemical shifts of the anomeric protons of Gal 6 and 6' [asialo-compound : 4.470 ppm], GlcNAc 5 and 5' [4.581 ppm] and Man 4 and 4' [5.119 and 4.926 ppm respectively] (17). Since the chemical shifts of the H-1 protons of Man 4 and 4' are different, their values can be used to assign the position of a (2→6) bound NeuAc residue in a mono-sialo bi-antennary structure. It should be noted that the only anomeric proton which undergoes a significant shift upon attachment of a NeuAc residue to the C-3 position of Gal, is the H-1 of Gal itself (17).
A highly significant pattern of signals is that of the Man H-2 protons [δH-2 Man 3 = 4.254 ppm, δH-2 Man 4 = 4.195 ppm and δH-2 Man 4' = 4.114 ppm]. This pattern gives direct information on the mannotriose branching region in the molecule to which at least the GlcNAc's 2, 5 and 5' are attached(16,25).
The singlet of the CH_3-protons of the N-acetyl group of GlcNAc 1 is found at δ = 2.007 ppm, that of GlcNAc 2 at δ = 2.080 ppm. Those of GlcNAc 5 and 5' coincide at δ = 2.030 ppm, whereas the CH_3's of the N-acetyl groups of both α(2→6) linked NeuAc residues resonate at 2.067 ppm. In case a NeuAc residue is linked α(2→3) to Gal, the positions of the N-acetyl signals of the GlcNAc's are unaltered, whereas that of the NeuAc residue shifts to 2.046 ppm (17,21). In the asialo-analogue of compound I the N-acetyl protons of GlcNAc 5 and 5' resonate at 2.050 ppm and 2.047 ppm, respectively. The resonance positions of the N-acetyl signals of GlcNAc 1 and 2 are not influenced by the presence or absence of sialic acid at the end of the chain.

As mentioned before, the bi-antennary structure can be conceived as a basic element of carbohydrate structures of the lactosamine type. Extension of this structure with additional N-acetyllactosamine units, β(1→4) attached to Man 4 and/or β(1→6) to Man 4', influences the chemical shifts of several reporter groups. In particular the sets of chemical shift values for the H-1 and H-2 protons of Man 3, 4 and 4' are characteristic for each type of these extended structures (10,20). One or more of the lactosamine units may bear a NeuAc residue in α(2→3) or α(2→6) linkage to Gal. When α(2→6) linked sialic acid occurs in one or more of these antennas, additional effects on the chemical shifts of the anomeric protons of the Man residues 4 and/or 4', can be expected [*vide supra*]. Due to the good resolution of the N-acetyl signals in the 360-MHz ^{1}H-NMR spectra even of these extended structures, their chemical shifts can also be used for recognizing the various antennary types of structure (10,20).
The H-3 and H-4 protons of Gal are useful structural reporter groups too. If a substituent like sialic acid is α-linked to the C-3 position of Gal, the H-3 of this Gal becomes well separated from the bulk of the non-anomeric protons : δH-3 = 4.120 ppm. Interestingly, if a substituent is linked β(1→3) to Gal the H-4 of the substituted Gal undergoes a characteristic shift : δH-4 : 3.91 ppm → 4.16 ppm [*cf.* (25)].
The extension of the lactosamine type structures with one or more Fuc residues has so far found to be occurring in α(1→3) and α(1→6) type of binding. The latter type of linkage has only been demonstrated when the Fuc residue is attached to GlcNAc 1. About this Fuc the NMR spectrum gives the following information:

a) recognition of the presence of Fuc by δH-1 = 4.89 ppm [$J_{1,2}$ = 3.8 Hz], δH-5 = 4.12 ppm and δH-6 = 1.21 ppm
b) location in the carbohydrate chain by the increments in chemical shift of H-1 of GlcNAc 2 [4.616 ppm → 4.683 ppm] and of the N-acetyl signal of GlcNAc 2 [2.076 ppm → 2.094 ppm] (21).

Fuc can be present in an α(1→3) linkage to one or more of the GlcNAc's of the lactosamine units (10,18). Typical NMR data for this type of residue are the following:

a) δH-1 = 5.11 ppm [$J_{1,2}$ = 3.8 Hz], δH-5 = 4.83 ppm and δH-6 = 1.17 ppm
b) the shift of the N-acetyl signal of the GlcNAc residue to which Fuc is attached [*e.g.* in case of attachment to GlcNAc 7 : 2.078 ppm → 2.064 ppm].

Recently we had the opportunity to widen the scope of our ^{1}H-NMR method for the identification of carbohydrate structures to several types of O-glycosidically linked carbohydrate chains. The physiological significance of the O-glycosidically linked chains is generally recognized (26,27). Also for this type of carbohydrate chains controversies exist about the primary structure of the sugar moiety of one and the same glycoprotein, *e.g.* pig submaxillary

gland mucin glycoprotein [PSM] (28,29,30). This illustrates again the necessity and relevance of the development of reliable and fast methods for the determination of these structures.
As a first example of this type of structures, we should like to discuss the ^{1}H-NMR spectral features of compound II: Galβ(1→4)Xylβ(1→O)Ser. This compound II is a part of the core of the linkage region between the polysaccharide and protein chain in several animal proteoglycans:

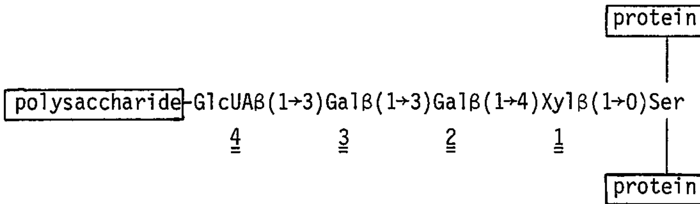

Fig. 4. Carbohydrate-protein linkage region in the protein complexes of heparin and chondroitin-4-sulfate, and numbering of the sugar residues.

The 360-MHz ^{1}H-NMR spectrum and structure formula of compound II are given in Fig. 5.

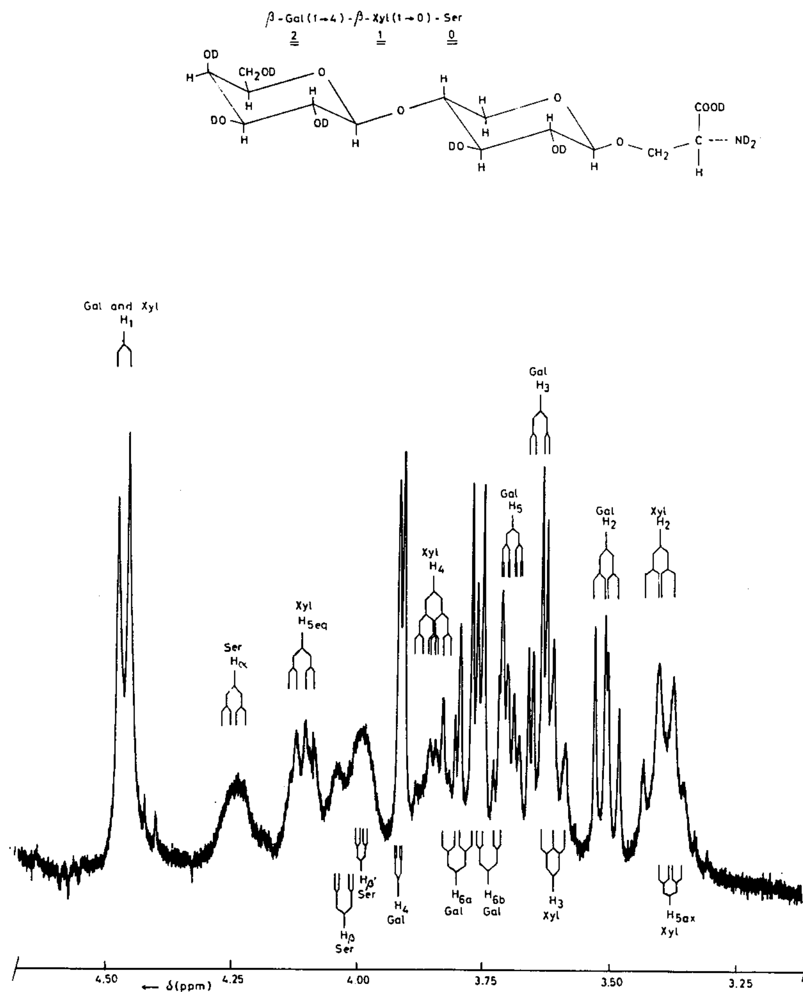

Fig. 5. 360-MHz ^{1}H-NMR spectrum of compound II in deuterium oxide.

The signals in the spectrum of compound II can be divided into groups in an analogous way as for the N-glycosidically linked carbohydrate chains, *viz.*

i) the bulk of the non-anomeric protons [in the region δ = 3.5–4.0 ppm] , which could be interpreted completely

ii) structural reporter groups

a) the anomeric protons

b) the H-5eq proton of the Xyl residue

c) the H-4 proton of the Gal residue

iii) the protons of the amino acid.

The resonances of the anomeric protons of the Xyl $\underline{\underline{1}}$ and Gal $\underline{\underline{2}}$ residue in compound II coincide at δ = 4.469 ppm. The β-type of linkage of both residues can be derived from the value of the coupling constants [$J_{1,2}$ = 8.0 Hz]. The substitution of Xyl by Gal in β(1→4) linkage is clearly reflected by a shift increment of the H-5eq proton of the Xyl residue [3.97 ppm → 4.12 ppm] It has to be expected that also in larger compounds this proton resonates outside of the bulk. The H-4 of a Gal residue terminating the chain as holds for compound II, resonates at δ = 3.92 ppm. Upon extension of compound II with a Gal *via* a β(1→3) linkage, the H-4 of the penultimate Gal shifts towards δ = 4.19 ppm.

In general, the isolation of glycopeptides consisting of O-glycosidically linked carbohydrate chains and only one or two amino acids is rather cumbersome. However the corresponding alditols of these carbohydrate chains can easily be obtained by alkaline borohydride reductive cleavage of the glycoproteins [-peptides]. As we could show, these alditols are excellent alternatives for glycopeptides, although information on the configuration of the carbohydrate-protein linkage is lost. The same type of reporter groups can be applied for the structural interpretation of the NMR spectra of these alditols, while the reduced monosaccharide offers some new parameters.

Several types of O-glycosidically linked carbohydrate chains, occurring in a wide variety of glycoproteins, have the following structural element in common:

Fig. 6. Characteristic carbohydrate-protein linkage region of mucus glycoproteins.

The ^{1}H-NMR spectrum of the corresponding alditol III of this compound is given in Fig. 7.

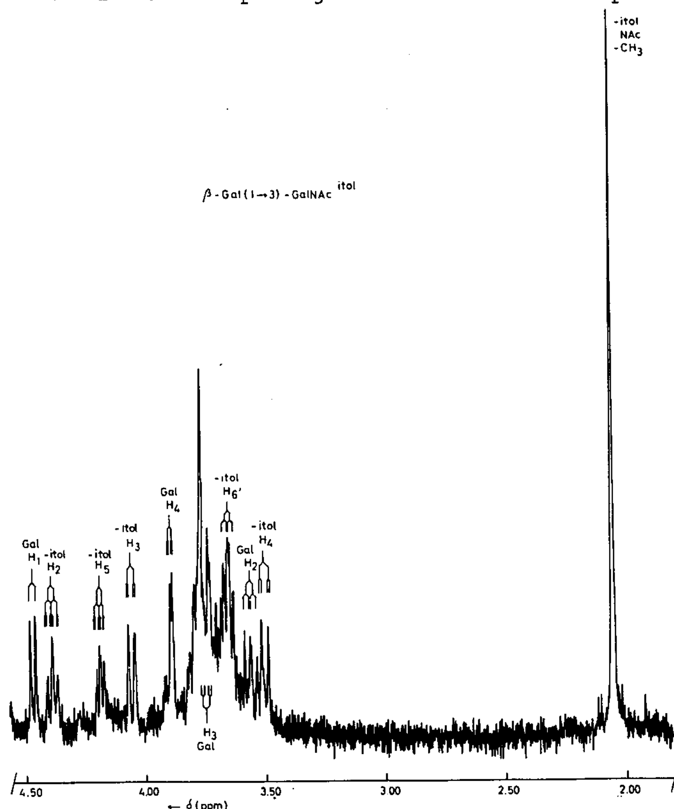

Fig. 7. 360-MHz ^{1}H-NMR spectrum of Galβ(1→3)GalNAc^{itol} in deuteriumoxide.

An almost complete interpretation of the spectrum of compound III could be achieved, as indicated in Fig. 7. The value of the coupling constant $J_{1,2}$ of Gal [7.3 Hz] illustrates the β-type of glycosidic linkage. Furthermore, the H-2, -3, -4 and -5 protons of the alditol resonate outside of the bulk. It can be expected that one or more of these protons are suitable as reporter groups *e.g.* in case of further substitution of $GalNAc^{itol}$.
The core structure Galβ(1→3)$GalNAc^{itol}$ has found to be enlarged with several types of monosaccharides in a variety of positions of attachment, thereby representing a wide divergency of in nature occurring carbohydrate chains. Extension with NeuAc in an α(2→3) linkage to Gal gives the structure of compound IV: NeuAcα(2→3)Galβ(1→3)$GalNAc^{itol}$. The 360-MHz ^{1}H-NMR spectrum of compound IV is presented in Fig. 8.

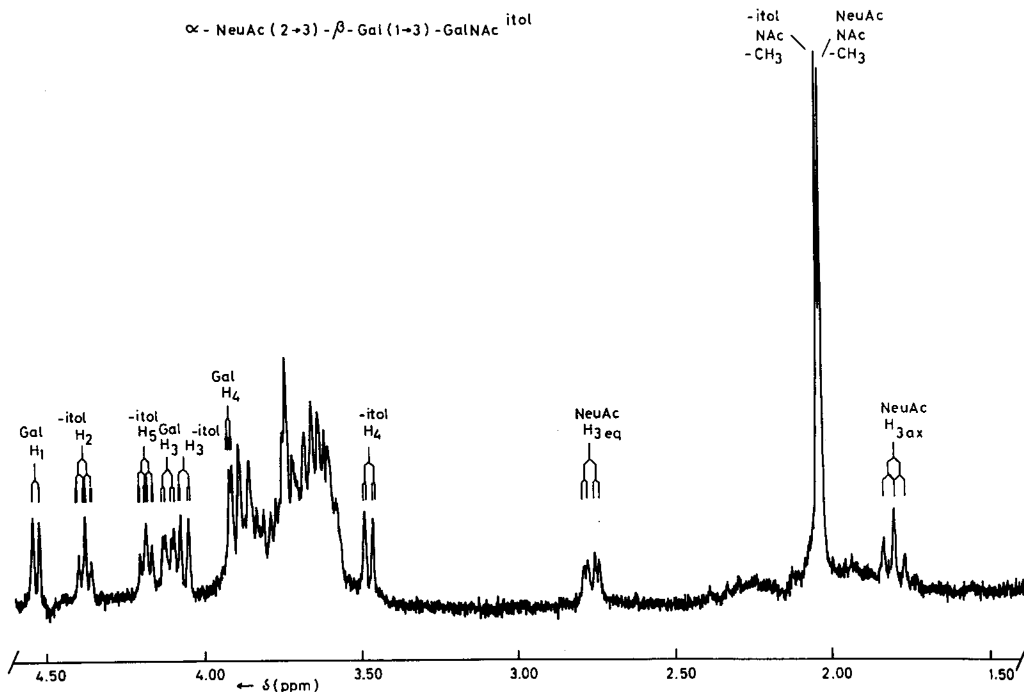

Fig. 8. 360-MHz ^{1}H-NMR spectrum of compound IV in deuteriumoxide.

Comparison of the spectrum of compound IV with that of the disaccharide-alditol III reveals the following structural reporter groups:

a) the anomeric proton of Gal; this undergoes a downfield shift [δH-1 : 4.475 ppm → 4.546 ppm], in accordance with earlier observations [*c f.* (17)].
b) the H-3 proton of Gal; substitution of the Gal residue by a monosaccharide α-linked to position C-3 leads to a shift of the resonance signal of H-3 from δ = 3.754 ppm to δ = 4.123 ppm [*vide supra*].
c) the H-3 protons of NeuAc; the set of chemical shift values δH-3ax = 1.802 ppm; δH-3eq = 2.770 ppm, is characteristic for the primary structure.
Attachment of NeuAcα(2→3) linked to Gal has virtually no influence on the chemical shifts of the $GalNAc^{itol}$-protons, except on that of
d) the N-acetyl group protons of $GalNAc^{itol}$, demonstrating a small but significant shift increment.

In case compound IV bears a NeuAc residue α(2→6) linked to $GalNAc^{itol}$, the following tetrasaccharide-alditol [compound V] arises: NeuAcα(2→3)Galβ(1→3)$GalNAc^{itol}$(6←2)αNeuAc.
The additional NeuAc residue is characterized by its specific set of chemical shift values of its H-3 protons [δH-3ax = 1.699 ppm; δH-3eq = 2.723 ppm]. Its presence affects the chemical shifts of the H-4, -5 and -6' of $GalNAc^{itol}$ [δH-4 : 3.494 ppm in compound IV → 3.517 ppm in compound V; δH-5 : 4.191 ppm → 4.246 ppm and δH-6' = 3.65 ppm → 3.470 ppm]. The signal of H-6' of $GalNAc^{itol}$ is now situated apart from the bulk, which makes it

characteristic for substitution at C-6 of the GalNAc^{itol}. Furthermore, attachment of NeuAcα(2→6) to GalNAc^{itol} brings about a specific shift increment of the N-acetyl signal of the alditol, compared with compound IV.

In another series of compounds the disaccharide-alditol core has found to be enlarged with a Fuc residueα(1→2) linked to Gal. In this way compound VI arises : Fucα(1→2)Galβ(1→3)GalNAc^{itol}. The 360-MHz ^{1}H-NMR spectrum of compound VI is given in Fig. 9.

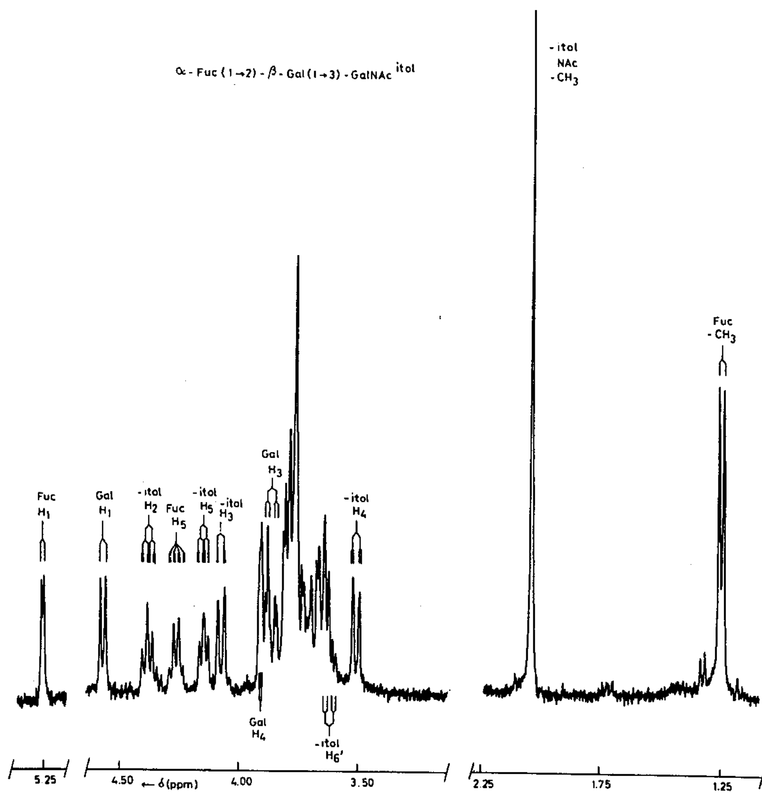

Fig. 9. 360-MHz ^{1}H-NMR spectrum of compound VI in deuteriumoxide.

Comparing the spectrum of compound VI with that of the afuco-compound III, on the structural reporter groups the following features have to be mentioned:

a) the chemical shift of the H-1 of the β-linked [$J_{1,2}$ = 7.5 Hz] Gal residue is 4.584 ppm, showing a downfield shift; the chemical shift of the H-1 of Fuc is 5.256 ppm. In accordance with the α-type of glycosidic linkage the value of its coupling constant $J_{1,2}$ is 4.0 Hz.
b) the chemical shift of H-5 of Fuc is 4.279 ppm, while that of the CH_3 group of Fuc is 1.243 ppm.
c) the attachment of Fuc to Gal gives rise to shift increments of H-3, -4 and -5 of GalNAc^{itol} [δH-3 : 4.062 ppm → 4.089 ppm; δH-4 : 3.506 ppm → 3.520 ppm; δH-5 : 4.198 ppm → 4.162 ppm].

Extension of the latter compound with a GalNAc residue α(1→3) linked to Gal affords compound VII : GalNAcα(1→3)[Fucα(1→2)]Galβ(1→3)GalNAc^{itol}. The attachment of this residue has a large influence on the NMR spectrum of the compound:

a) the signal of the anomeric proton of the additional GalNAc is found at δ = 5.389 ppm [$J_{1,2}$ = 2.4 Hz, indicative of an α-type of glycosidic linkage between GalNAc and Gal], while the H-1 of Gal is shifted downfield to δ = 4.720 ppm, and the H-1 of Fuc upfield to δ = 4.189 ppm.
b) as mentioned above, a residue α-linked to the C-3 position of Gal causes a downfield shift of the H-3 proton of this Gal : in the spectrum of compound VII the H-3 proton of Gal resonates at δ = 4.254 ppm. This extremely downfield position is a result of a cooperativity of Fuc and GalNAc.
c) the signal of the H-5 proton of Fuc is shifted from δ = 4.279 ppm to 4.343 ppm; the position of the CH_3-protons of Fuc is not influenced.

d) shift increments are observed for the H-2, -3, -4 and -5 protons of GalNAc^{itol} [δH-2 : 4.398 ppm → 4.302 ppm; δH-3 : 4.089 ppm → 4.100 ppm; δH-4 : 3.520 → 3.606 ppm; δH-5 : 4.162 ppm → 4.125 ppm].

Unambiguous assignment of the signals of the N-acetyl group protons has not been carried out so far.
Attachment of NeuGlα(2→6) to GalNAc^{itol} yields the pentasaccharide-alditol VIII : GalNAcα(1→3)[Fucα(1→2)]Galβ(1→3)GalNAc^{itol}(6←2)αNeuGl.
In the spectrum of this compound, the presence of the NeuGl residue comes to expression in the typical set of chemical shift values of its H-3 protons [δH-3ax = 1.711 ppm; δH-3eq = 2.750 ppm] and in the singlet from the CH_2-group protons of the N-glycolyl function at δ = 4.123 ppm. The magnitudes of the effects of NeuGl on the chemical shifts of the GalNAc^{itol} reporter groups are comparable with those of the attachment of NeuAcα(2→6) linked to GalNAc^{itol} [compound III → IV]. The chemical shifts of all other reporter groups remain unchanged.

This study has shown that the NMR spectral features of each compound mentioned above, are characteristic. They can be applied in the structure elucidation of glycoconjugates. So far mainly chemical shifts and coupling constants have been used, but it can be assumed that also other NMR-parameters *e.g.* relaxation times, can provide useful information. Further progress can be expected from the application of newer NMR-techniques like two-dimensional NMR as well as from instruments operating at higher magnetic fields.

Abbreviations –

NMR	Nuclear Magnetic Resonance	Fuc	fucose
Asn	asparagine	GlcUA	glucuronic acid
Ser	serine	GlcNAc	N-acetylglucosamine
Thr	threonine	GalNAc	N-acetylgalactosamine
Xyl	xylose	NeuAc	N-acetylneuraminic acid
Gal	galactose	NeuGl	N-glycolylneuraminic acid
Man	mannose	GalNAc^{itol}	N-acetylgalactosaminitol

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