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HIGH RESOLUTION ^{1}H-NMR SPECTROSCOPY OF CARBOHYDRATE STRUCTURES

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High resolution ^{1}H-NMR spectroscopy has obtained a firm position among the techniques available for structural studies of bio-molecules. The instrumentation has enormously been improved during the last decade. New methods have been developed to optimize the information which can be deduced from the spectra[1-5]. ^{1}H-NMR spectroscopy has provided valuable data on the structure, conformation and inter- and intra-molecular interactions of various types of bio-molecules.

Only recently, the application of high resolution ^{1}H-NMR spectroscopy to complex carbohydrates started. In the beginning mainly ^{1}H-NMR data were described for derivatives of mono- and oligosaccharides. We investigated by 220-MHz and/or 300-MHz ^{1}H-NMR pertrimethylsilyl[6-10] and permethyl derivatives[11-14]. The application of derivatives has the drawback that chemical modifications have to be carried out, followed by sometimes elaborate purifications or fractionations. On the other hand, it has the advantage that for reducing compounds, the different anomeric forms can be isolated and separately investigated. For derivatives of relatively simple compounds it turned out to be possible to give a complete interpretation of the spectra. In general the definite assignment and refinement of the NMR-parameters could be obtained by spectral simulation in an iterative procedure. The resulting NMR data can be used to derive detailed information on many structural aspects like configuration of the glycosidic bonds, substitution pattern, type and conformation of the constituting monosaccharides. Going to larger oligomers, the spectra become more complex, thereby making a complete

interpretation virtually impossible. However, already a partial interpretation of the spectra can furnish relevant information which is difficult to obtain along other routes.

Similar interpretation problems as mentioned for derivatives are encountered in the study of free saccharides dissolved in D_2O. In the case of reducing saccharides, there is the additional complication that anomeric mixtures have to be investigated unless a chemical modification of the reducing end is carried out. In the following a few examples will be presented of the scope and limitations of 360-MHz ^{1}H-NMR spectroscopy of carbohydrate structures. In the spectra the signals can roughly be subdivided into resonances of:

a. the anomeric protons
b. the bulk of the non-anomeric protons, which usually gives rise to a broad, poorly resolved signal
c. special non-anomeric protons, which resonate outside of "the big hump". These important signals can be conceived as "structural reporter groups".
d. protons of substituents like N-acetyl, N-glycolyl, O-acetyl, O-lactyl groups etc.

Let us first consider the simple disaccharide β-Gal*p*(1→3)-Gal, which gives rise to a highly complex spectrum as shown in Fig. 1.

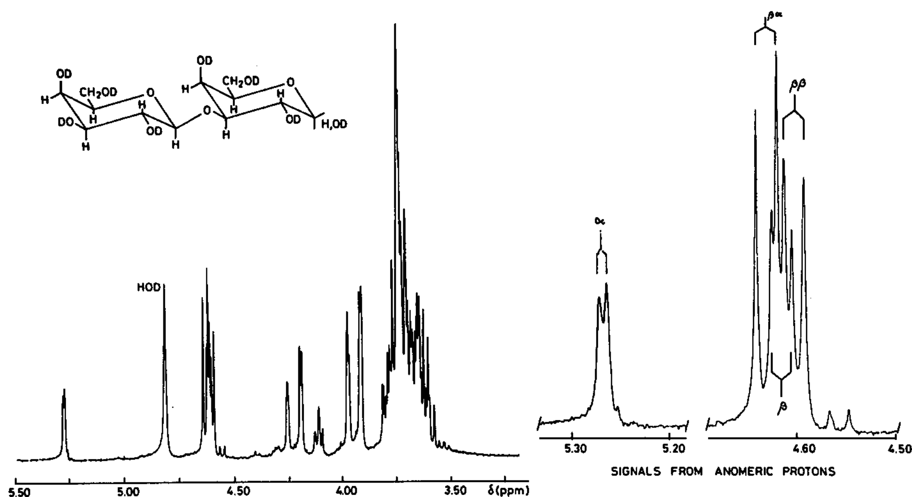

Fig. 1. 360-MHz ^{1}H-NMR spectrum of β-Gal*p*(1→3)-Gal in D_2O

This spectrum is mainly a superposition of the spectra of β-Gal*p*(1→3)-α-Gal*p* and β-Gal*p*(1→3)-β-Gal*p*. As indicated in Fig. 1, the anomeric signals can easily be recognized. It has to be noted that differences in anomeric configuration at the reducing end are also reflected in the chemical shifts in all of the skeleton protons of the reducing unit and in some of the ring protons of the non-reducing unit.

The spectrum of the alditol obtained after reduction with $NaBH_4$ of β-Gal*p*(1→3)-Gal is much simpler and can be interpreted in more detail as demonstrated in Fig. 2.

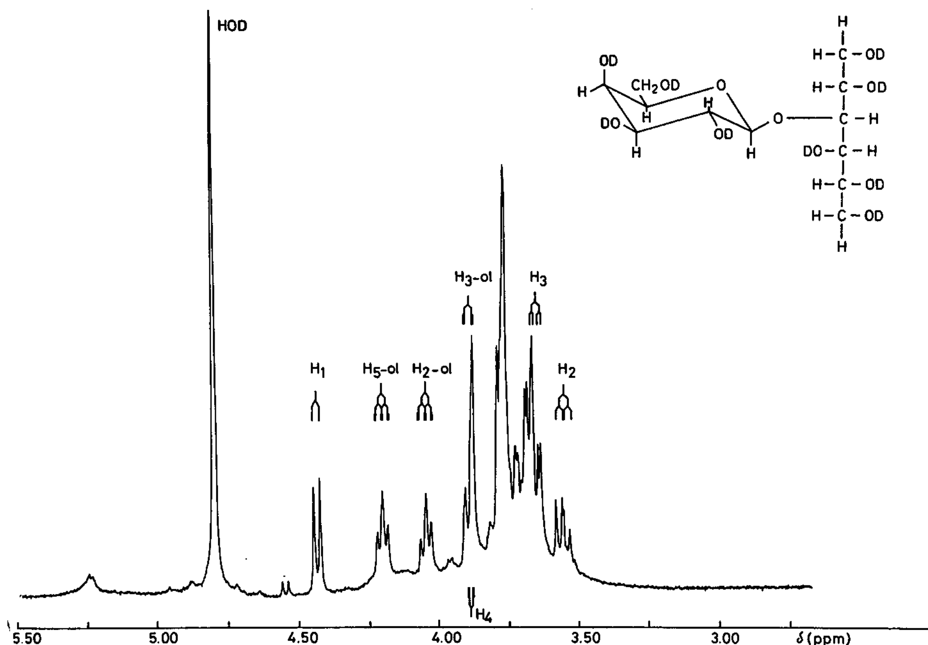

Fig. 2. 360-MHz ^{1}H-NMR spectrum of β-Gal*p*(1→3)-Gal-ol in D_2O

The substitution of galactitol and the configuration of the glycosidic linkage can be derived from the NMR data. Spectra of reducing disaccharides are not always as complicated as given in Fig. 1. Anomerization does not necessarily affect the chemical shifts of so many protons. For example the spectrum of lactose as shown in Fig. 3 is somewhat less complex. The resonance position of the H-1 of the non-reducing galactose residue is identical for both anomers.

As mentioned before, for larger compounds only a partial interpretation of the spectrum can be achieved.

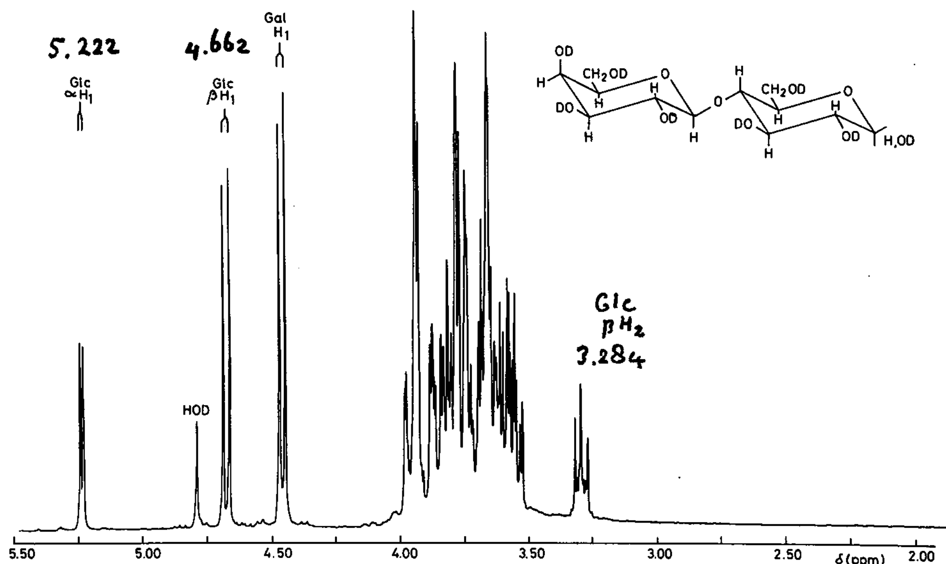

Fig. 3. 360-MHz ^{1}H-NMR spectrum of β-Gal*p*(1→4)-Glc (= lactose) in D_2O

As indicated in the spectra of the trisaccharides α-NeuAc*p*(2→3)--β-Gal*p*(1→4)-Glc (Fig. 4) and α-NeuAc*p*(2→6)-β-Gal*p*(1→4)-Glc (Fig. 5) the anomeric protons and the H-3 protons of sialic acid resonate at positions distinctly different from those of the bulk. Interestingly, the chemical shifts of the H-3 protons of sialic acid are characteristic for the position and configuration of the linkage of NeuAc to Gal. To some extent the chemical shifts of the H-3 protons are also influenced by the type of sugar residue to which Gal is glycosidically attached. E.g. replacement of Glc by GlcNAc introduces shift increments as shown in Table 1. On the other hand, the chemical shifts of H-1 of Gal are significantly affected by the presence and the position of attachment of sialic acid as demonstrated in Table 2.

The chemical shifts of the H-3 protons of sialic acid are extremely useful in structural studies. For example in horse pancreatic ribonuclease we have found that (2→3) as well as (2→6) linked sialic acid residues can occur in one glyco chain (Fig. 6).

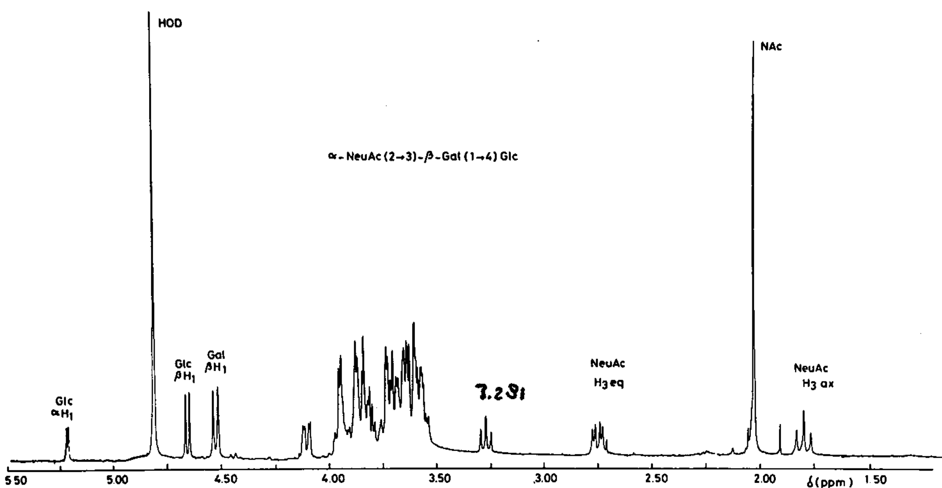

Fig. 4. 360-MHz ^{1}H-NMR spectrum of α-NeuAc*p*(2→3)-β-Gal*p*(1→4)-Glc (= sialyl(2→3)lactose) in D_2O

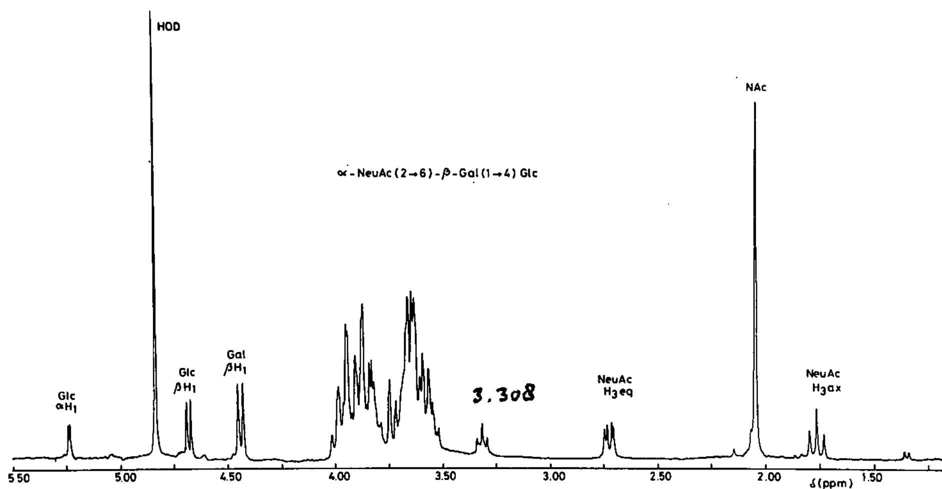

Fig. 5. 360-MHz ^{1}H-NMR spectrum of α-NeuAc*p*(2→6)-β-Gal*p*(1→4)-Glc (= sialyl(2→6)lactose) in D_2O

Table 1. Chemical shifts of sialic acid H-3 protons for various sialic acid glycosides*

Compound	δH-3ax	δH-3eq
α-NeuAc Methylglycoside[15]	1.626	2.718
β-NeuAc Methylglycoside	1.645	2.337
α-NeuAc(2→3)-β-Gal(1→4)-Glc[16]	1.799	2.757
α-NeuAc(2→6)-β-Gal(1→4)-Glc[16]	1.739	2.712
α-NeuAc(2→3)-β-Gal(1→4)-β-GlcNAc-1→R[16]	1.800	2.758
α-NeuAc(2→6)-β-Gal(1→4)-β-GlcNAc-1→R[16]	1.721	2.670

* ^{1}H Chemical shifts for neutral solutions in D_2O; ppm relative to DSS.

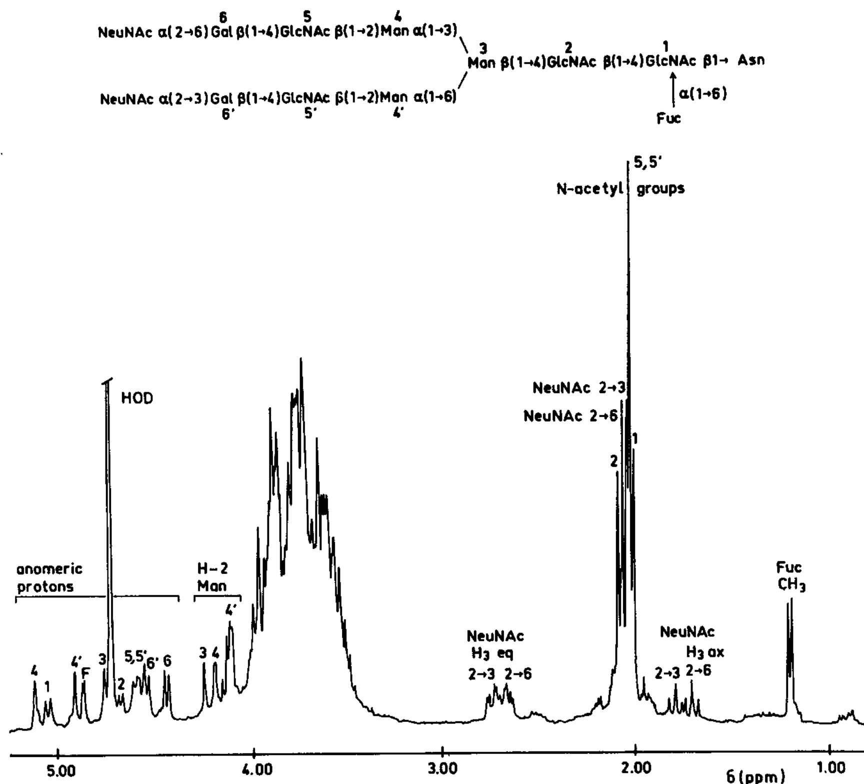

Fig. 6. 360-MHz ^{1}H-NMR spectrum of glycopeptide Eq. GPI isolated from horse pancreatic ribonuclease.

Table 2. Chemical shifts of the galactose H-1 proton for lactose, sialyl(2→3)lactose and sialyl(2→6)lactose

Compound	δH-1 Gal
β-Gal(1→4)-Glc	4.443
α-NeuAc(2→3)-β-Gal(1→4)-Glc[16]	4.531
α-NeuAc(2→6)-β-Gal(1→4)-Glc[16]	4.427

On the basis of the peak areas and the chemical shifts of the H-3 protons, the molar ratios of the (2→3) and (2→6) linked sialic acids could be derived[17]. The location of the differently linked sialic acids could be inferred from the chemical shifts of the H-1 protons of the mannose residues (vide infra).

For compounds containing the mannotriosido branching core as indicated in Fig. 7 the H-2 protons of mannose can act as structural reporter groups.

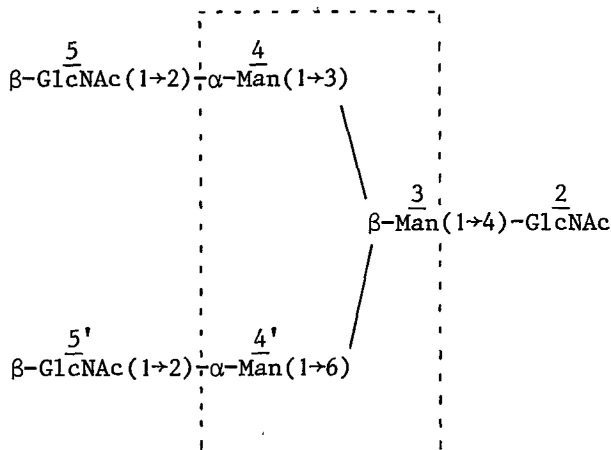

Fig. 7. Structure of a hexasaccharide (A), possessing the mannotriosido branching core (within dotted lines).

This oligosaccharide is a structural unit of many N-glycosidically linked carbohydrate chains of glycoproteins. In Fig. 8 the most relevant part of the spectrum is given[18].

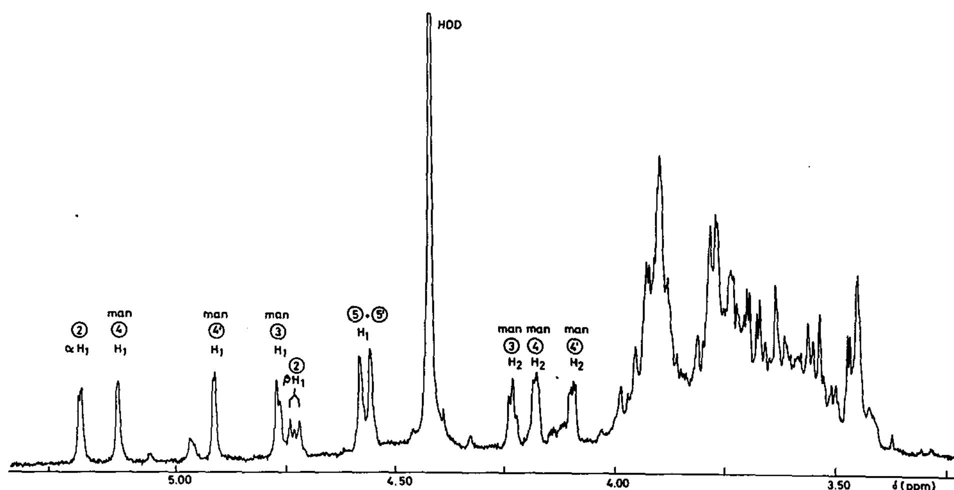

Fig. 8. 360-MHz ^{1}H-NMR spectrum of hexasaccharide A (see Fig. 7) in D_2O

The set of H-2 protons of mannose has not only characteristic chemical shifts, but the shape of the signals is also typical. Changes in the substitution pattern of the mannotriose unit are reflected in shift increments of one or more of the H-1 and H-2 protons of the mannose residues as summarized in Table 3[19] for the lactosamine type of carbohydrate chains (see Fig. 9 for structures).

Sialic acid residues α(2→6) linked to Gal in carbohydrate chains of the lactosamine type (compare Fig. 6) have a long distance effect on the H-1 of the mannose residue occurring in the same branch. Such an effect is absent for α(2→3) linked sialic acid residues. This feature has opened the possibility to determine the location of these residues in compounds wherein both types of sialic acid linkages occur. This approach is effective because the chemical shifts of the H-1 protons of the various mannose residues in the asialo compounds are different (Table 4) [17,19]. Evidently the shift increments of the H-1 of Gal and GlcNAc from the lactosamine units cannot be used because the chemical shifts of the H-1 protons are almost identical for the different branches.

```
   6         5          4
βGal(1→4)βGlcNAc(1→2)αMan(1→3)
                              \ 3        2          1
bi-antenna                    βMan(1→4)βGlcNAc(1→4)βGlcNAc1→Asn
   6'        5'         4'    /
βGal(1→4)βGlcNAc(1→2)αMan(1→6)

   8         7
βGal(1→4)βGlcNAc(1→4)
   6         5      \   4
βGal(1→4)βGlcNAc(1→2)αMan(1→3)
                              \ 3        2          1
tri-antenna                   βMan(1→4)βGlcNAc(1→4)βGlcNAc1→Asn
   6'        5'         4'    /
βGal(1→4)βGlcNAc(1→2)αMan(1→6)

   8         7
βGal(1→4)βGlcNAc(1→4)
   6         5      \   4
βGal(1→4)βGlcNAc(1→2)αMan(1→3)
                              \ 3        2          1
tetra-antenna                 βMan(1→4)βGlcNAc(1→4)βGlcNAc1→Asn
   6'        5'         4'    /
βGal(1→4)βGlcNAc(1→2)αMan(1→6)
   8'        7'     /
βGal(1→4)βGlcNAc(1→6)

             5          4
          βGlcNAc(1→2)αMan(1→3)
                              \ 3        2
hexasaccharide A              βMan(1→4)GlcNAc
             5'         4'    /
          βGlcNAc(1→2)αMan(1→6)

             5          4
          βGlcNAc(1→2)αMan(1→3)
                      9       \ 3        2
heptasaccharide B  βGlcNAc(1→4)βMan(1→4)GlcNAc
             5'         4'    /
          βGlcNAc(1→2)αMan(1→6)
```

Fig. 9. Structures of the asparagine-linked glycans of the N-acetyllactosamine type, hexasaccharide A and heptasaccharide B.

Table 3. Chemical shifts of mannose H-1 and H-2 protons for bi-, tri- and tetra-antennary asparagine-bound glycan chains of the N-acetyllactosamine type, and for oligosaccharides A and B (see Fig. 9).

Structure	δH-1 of residue			δH-2 of residue		
	3	4	4'	3	4	4'
bi-antenna	4.764	5.121	4.928	4.247	4.189	4.110
tri-antenna	4.757	5.119	4.924	*4.215*	*4.215*	4.109
tetra-antenna	4.754	5.127	*4.866*	*4.215*	*4.215*	*4.092*
hexasaccharide A	∿4.77	5.119	4.922	∿4.25	4.192	4.112
heptasaccharide B	∿*4.70*	*5.062*	*5.004*	∿*4.18*	*4.250*	*4.151*

Table 4. Chemical shifts of mannose H-1 protons for an asialo-biantennary asparagine-bound glycan chain of the N-acetyllactosamine type (see Fig. 9), and of glycopeptide Eq. GPI from horse pancreatic ribonuclease (see Fig. 6).

Structure	δH-1 of residue		
	3	4	4'
asialo-biantenna	4.764	5.121	4.928
Eq. GPI	4.766	*5.135*	4.923

Table 5. Chemical shifts of H-1, H-5 and H-6 protons of fucose in different linkages to N-acetylglucosamine.

type of linkage	δH-1	δH-5	δH-6
α-Fuc(1→3)-β-GlcNAc(1→	5.11	4.83	1.17
α-Fuc(1→4)-β-GlcNAc(1→	5.02	4.87	1.18
α-Fuc(1→6)-β-GlcNAc(1→	4.90	4.12	1.21

Another example of a reporter group is the H-5 proton of fucose. In the carbohydrate chains of glycoproteins various types of fucose linkages can occur. As shown in Table 5, the α(1→3)-, α(1→4)- and α(1→6)-linkage of fucose to N-acetylglucosamine can be distinguished on the basis of the chemical shifts of the H-5 and H-1 protons of fucose[20,21].

Apart from this structural information, which can be derived from the spectra, it is important to stress that the spectra are useful for determination of the homogeneity of the sample and of the quantitative carbohydrate composition of pure compounds. These apparently trivial data are not always easy to obtain along classical routes. Structural studies on heterogeneous samples may lead to erroneous results. In view of the non-destructiveness of NMR investigations, these types of experiments have to be carried out before chemical and/or enzymic degradations are performed.

With regard to the ^{1}H-NMR studies of intact, underivatized gangliosides we are still in a very preliminary stage. An extremely important aspect is the choice of the solvent. As shown in Fig. 10, it is evident that D_2O is unsuitable, due to the formation of aggregates.

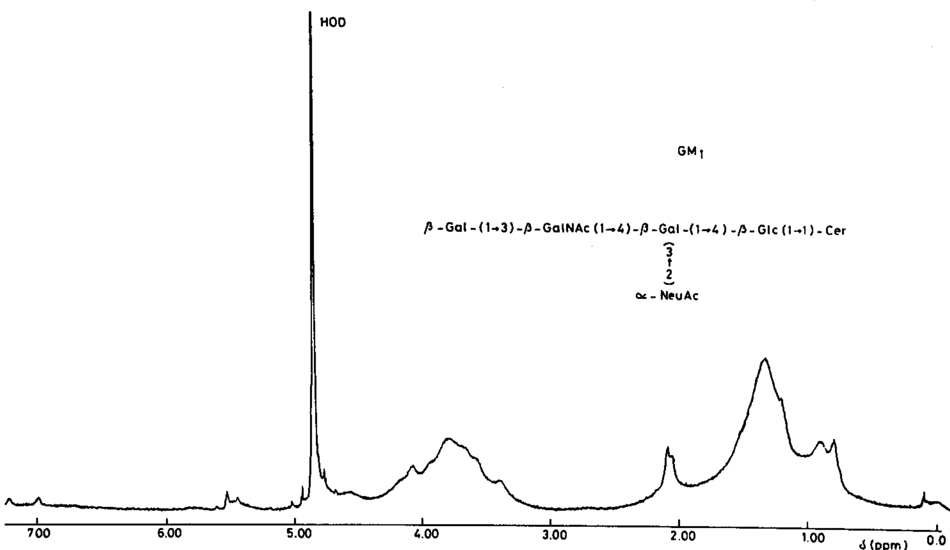

Fig. 10. 360-MHz ^{1}H-NMR spectrum of ganglioside GM_1 in D_2O

The spectrum provides hardly any structural information. However, it seems that spectra recorded in DMF-d_7 (Fig. 11) can yield valuable data. The resolution of the significant signals is at least comparable to that of the permethyl compounds[22-24].
It is likely, that also for the analysis of gangliosides high resolution ^{1}H-NMR spectroscopy can become a very powerful tool.

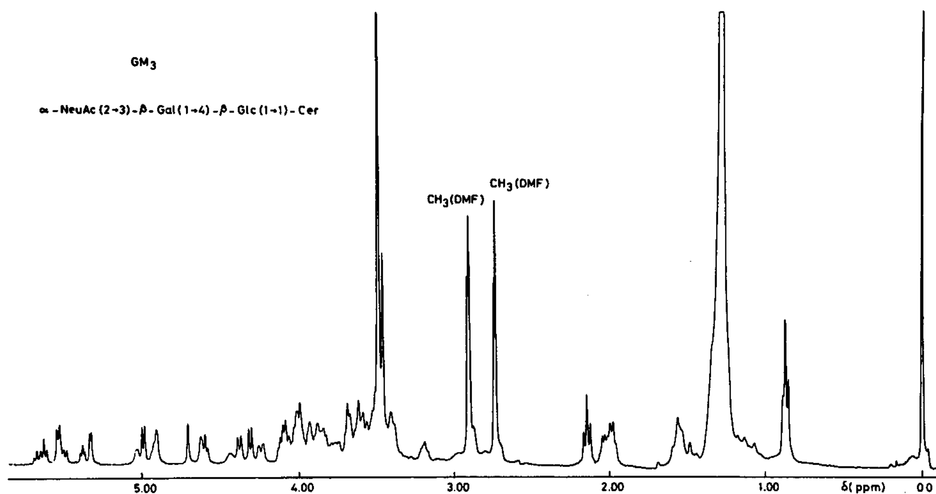

Fig. 11. 360-MHz ^{1}H-NMR spectrum of ganglioside GM_3 in DMF-d_7

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