Carbohydrate Structural Determination by NMR Spectroscopy: Modern Methods and Limitations†

Jens Ø. Duus, Charlotte H. Gotfredsen, and Klaus Bock*

Carlsberg Laboratory, Department of Chemistry, Gamle Carlsberg Vej 10, DK-2500 Valby Copenhagen, Denmark

Received July 17, 2000

Contents

I. Introduction and Scope

Carbohydrates play key roles in biological recognition processes,[1,2] in development of diseases,[3] and in many important areas of food and technical industry. The function of these molecules and detailed mechanisms of these events are still poorly understood. As such, carbohydrates remain the least exploited among the three major classes of biomolecules. Carbohydrates are difficult to synthesize and manipulate,[4] and the limited availability makes it difficult to study their biological functions in detail. There is, e.g., not yet a machine available commercially which can synthesize complex oligosaccharides in an automated fashion. Biologists do not have a PCR method available for carbohydrates to facilitate structural assignment. In addition, there is only limited experience in the preparation of glycoproteins with well-defined carbohydrate structures to investigate their roles in glycoprotein structure and function. Medicinal chemists have doomed complex carbohydrates as uninteresting in drug development, since carbohydrates larger than a disaccharide are generally too complex to be prepared in sufficient amounts for testing and too hydrophilic to have good bioavailability. Furthermore, they are generally orally inactive and easily degraded in the digestive tract.

It is, however, important to encourage and continue the study of carbohydrate-mediated biological processes and to investigate their behavior, as understanding the mechanism of carbohydrate function may lead to the development of carbohydrate-based therapeutics. Recent advances in glycochemistry have helped to solve some of the problems associated with studies of carbohydrates, and methods for the large-scale synthesis of complex carbohydrates for drug development have been documented. Current efforts are, however, directed toward the development of small molecules to mimic the structure and function of complex carbohydrates with the hope that such compounds can be more easily prepared.

This review will address the general problem of structural assignment of complex carbohydrate structures by NMR spectroscopy. This is a field which during the past decades has demonstrated its vast importance in all the above-mentioned fields, and this is expected to continue in the future. The study of sugar nucleotides and nucleosides will not be covered.

The building blocks in oligosaccharides are more diverse in nature than in proteins or nucleic acids. Carbohydrates often differ only from each other in the stereochemistry, and the pattern of interresidue linkages can be very heterogeneous.[5] The information capacity of the carbohydrates is much larger than in proteins, particularly due to branched structures. It has been claimed that carbohydrates contain the hidden code to biological recognition.[5−7] Consequently, the structure determination of complex oligosaccharides is a challenging but difficult problem.

Carbohydrates with important biological functions present on, e.g., the protein surface[2,8] are often large structures, and NMR spectroscopy is one technique for their description. However, a wealth of techniques [9−19] are currently being applied in the identification of known oligosaccharides or the determination of new structures. The identification of known structures can be carried out on relatively small amounts of material using, e.g., capillary electrophoresis[10] or

† This paper is dedicated to the late Professor R. U. Lemieux in memory of an always exiting and stimulating collaboration on structural studies of oligosaccharides.

10.1021/cr990302n CCC: $35.00
Published on Web 11/15/2000

Jens Ø. Duus received his M.Sc. degree in Chemical Engineering from the Technical University of Denmark in 1989. Following this, he continued his graduate study with Professor Klaus Bock at the Carlsberg Laboratory and was granted his Ph.D. degree from the Technical University of Denmark in 1993. During his graduate study he was twice a Visiting Scientist at Dupont Experimental Station in Wilmington, DE working with Dr. S. Sabesan. From 1993 to 1994 he was a Research Fellow in Chemistry at the Beckman Institute at the California Institute of Technology working with Professor Harry B. Gray. After returning to the Carlsberg Laboratory, he was appointed Senior Scientist. His main fields of research are structure and dynamics of biomolecules with emphasis on carbohydrates using NMR and fluroescence.

Charlotte Held Gotfredsen received her M.Sc. degree in 1995 from Odense University, Denmark. She continued her graduate studies at Odense University with Dr. Jens Peter Jacobsen and Professor Jesper Wengel working on the synthesis and NMR structural studies of modified oligonucleotides and received her Ph.D. degree in Chemistry in 1998. During her Ph.D. study Charlotte spent a year with Professor Juli Feigon at the University of California, Los Angeles, CA. Since May 1998 she has been working as a Postdoctoral Fellow in the Department of Chemistry at the Carlsberg Laboratory in Copenhagen, Denmark. In 1999, Charlotte received a two-year personal grant (talent project) from the Danish Technical Research Council. Her current field of research is centered around the structural analysis of minute sample amounts and solid-phase bound compounds with emphasis on using the newly developed high-resolution MAS NMR probes and other microcoil NMR probes.

Klaus Bock is Professor at Carlsberg Laboratory, Department of Chemistry and Research Director at Carlsberg Research Center. After receiving his M.Sc. degree in Civil Engineering (Chemistry), 1968, he obtained his Ph.D. degree in Organic Chemistry, 1970, both at The Technical University of Denmark. He has been employed as Assistant Professor in the Department of Organic Chemistry, Technical University of Denmark in Lyngby (1970–1972), Associate Professor (Lecturer) in the Department of Organic Chemistry, Technical University of Denmark (1972–1988), Visiting Associate Professor, Department of Chemistry, Univeristy of British Columbia, Vancouver, B.C., Canada (1974–1975), Professor, Head of Chemistry Department, Carlsberg Laboratory, Copenhagen, Denmark (1988–present), Research Director, Carlsberg Research Center (1993–present), and Group Vice President for Research (1999–present). He has received several international awards and he has considerable representation on international committees, scientific advisory boards and journals, and boards. He has published over 256 refereed papers in international journals within the area of analytical and synthetic carbohydrate chemistry.

fluorescence detected HPLC.[9] Both techniques can be combined with mass spectrometry to give important structural information.[11,12]

An interesting technique has been developed for structure elucidation of glycoprotein-derived oligosaccharides based on the combined use of specific hydrolases and HPLC.[20] Mass spectrometry of oligosaccharides[11,16,21,22] is rapidly developing following improvements in instrumentation. The technique can in itself often lead to a full structural characterization of oligosaccharides. However, the crucial issue of sugar stereochemistry cannot be solved by current methodology used routinely in mass spectroscopy. The present review will therefore only include mass spectroscopy, when used in combination with NMR spectroscopy.

Carbohydrate NMR spectroscopy has continued to develop rapidly and has been reviewed several times during the last years (Table 1). Therefore, it seems most appropriate mainly to review the latest techniques and applications. The majority of previous reviews have focused on the application of NMR spectroscopy in the determination of three-dimensional structure or conformation of complex oligosaccharides (Table 1). In this field several new techniques have emerged, but they will only be described here if they have been used in the determination of the primary structure of carbohydrates or their derivatives. Many of the reviews which focus on structural determination are quite old but are included in Table 1 because they include useful data, either with description of methodology or reference to chemical shift data. The importance of publishing good NMR data for selected reference compounds can hardly be overestimated. This is clearly seen by searching the citation index for some of the older tabulations of chemical shifts. It will therefore be important that editors and publishers also allow the publication of what can seem to be trivial tables of chemical shifts eventually as supplementary information in electronic form in the future.

In the present review only a few examples of the large number of publications dealing with conformational analysis and binding studies of carbohydrates to protein receptors will be mentioned. This field has

Table 1. Reviews Related to Carbohydrates and NMR

author	title	year
Bush, C. A.; Martin-Pastor, M.; Imberty, A.	Structure and conformation of complex carbohydrates of glycoproteins, glycolipids, and bacterial polysaccharides	1999[23]
Roumestand, C.; Delay, C.; Gavin, J. A.; Canet, D.	A practical approach to the implementation of selectivity in homonuclear multidimensional NMR with frequency selective-filtering techniques. Application to the chemical structure elucidation of complex oligosaccharides	1999[24]
Poveda, A.; Jimenez-Barbero, J.	NMR studies of carbohydrate-protein interactions in solution	1998[25]
Schmeider, H.-J.; Hacket, F.; Rüdiger, V.	NMR studies of cyclodextrins and cyclodextrin complexes	1998[26]
Widmalm, G.	Physical methods in carbohydrate research	1998[27]
Hounsell, E. F.; Bailey, D.	Approaches to the structural determination of oligosaccharides and glycopeptides by NMR	1997[28]
Uhrín, D.	Concatenation of polarization transfer steps in 1D homonuclear chemical shift correlated experiments. Application to oligo- and polysaccharides	1997[29]
Agrawal, P. K.; Pathak, A. K.	Nuclear magnetic resonance spectroscopic approaches for the determination of interglycosidic linkage and sequence in oligosaccharides	1996[30]
Bush, C. A.	Polysaccharides & complex oligosaccharides	1996[31]
Lerner, L. E.	Carbohydrate structure and dynamics from NMR	1996[32]
Mulloy, B.	High-field NMR as a technique for the determination of polysaccharide structures	1996[33]
Peters, T.; Pinto, B. M.	Structure and dynamics of oligosaccharides: NMR and modeling studies	1996[34]
Van Halbeek, H.	Carbohydrates & glycoconjugates	1996[35]
Hounsell, E. F.	^{1}H NMR in the structural and conformational analysis of oligosaccharides and glycoconjugates	1995[36]
Van Halbeek, H.	NMR developments in structural studies of carbohydrates and their complexes	1994[37]
Abeygunawardana, C.; Bush, C. A.	Determination of the chemical structure of complex polysaccharides by heteronuclear NMR spectroscopy	1993[38]
Agrawal, P. K.	NMR spectroscopy in the structural elucidation of oligosaccharides and glycosides	1992[39]
Bush, C. A.; Cavas, P.	Three-dimensional conformations of complex carbohydrates	1992[40]

recently experienced an increase in new techniques after some years where the majority of conformational analyses from an experimental point of view were based primarily on quantification of NOEs. A new technique based on the measurement of residual dipolar coupling constants in partially aligned media, using bicelles or phage particles as cosolutes, has been introduced during the last years.[41−46] These parameters can give information not only about short-range interactions as NOEs, but also on the relative orientation of subunits or residues. Most likely a number of applications of this new technique will appear in the coming years. However, like in the use of NOEs in conformational studies of carbohydrates, the accurate interpretation of the residual dipolar coupling constants must take into account the internal flexibility as an important issue. During recent years, several studies have focused on the dynamic properties of carbohydrates, mainly based on the measurement of NMR relaxation parameters and among others ^{13}C data[47] combined with computer simulation.[48−50]

Solid-state NMR techniques are also being used to some degree, particularly within the field of polysaccharides, but it is beyond the scope of the present review to include such advances.[51−55]

Finally, this review will not cover routine applications of NMR in the structural elucidation of carbohydrates but will mainly focus on methodology using recent examples from the literature. This limitation is prompted by the large number of publications in the field, e.g., a search in Medline via PubMed in May 2000 using the keywords (carbohydrate* OR oligosaccharide* OR polysaccharide*) and NMR produced 6272 hits. Selecting from these only articles published in 1999, 366 hits were found. This search did not cover all relevant literature in the field because part of the chemistry-related literature is not listed in Medline. This interpretation is supported by the fact that a search in Science Citation Index, which is expected to cover all areas of research, for only 1999 resulted in 457 hits using the same search profile.

II. NMR Methods for Structural Determination

The first part of this section will introduce carbohydrate structural determination to the less experienced researcher or student in the field. The latter part will be focused on some more recently developed methods.

A. Carbohydrate Structural Elements and Classical NMR Methods

There are several ways to perform a primary structural analysis of a mono-, oligo-, or polysaccharide by NMR spectroscopy, and research groups performing carbohydrate structural determination have different approaches. Vligenthart et al.[56] introduced the 'structural-reporter-group' concept, which is based on signals outside the bulk region (3−4 ppm) in the ^{1}H NMR spectra of carbohydrates. This approach is used to identify individual sugars or sequences of residues and can be used to identify structural motifs or specific sugars and linkage compositions found in relevant databases, like CarbBank or SUGABASE.[57−59] Similarly, Kochetkov and co-workers developed a program and database which can assist in the assignments of oligo- and polysaccharides using primarily ^{13}C NMR data.[60−62] Furthermore, CASPER is a database tool constructed for

prediction of carbon and proton chemical shifts in combination with appropriate coupling constants for oligosaccharides. The predicted data are compared with experimental data and used to rule out irrelevant structures.[63−65] The computer programs used in structural determination will be described in more detail in section II.C. It is always recommended to check databases for characteristic chemical shifts and coupling constants of a given sequence. It will not necessarily solve the sequence composition completely but can be used to verify a sequence related to a known structure or a motif already present in a database.

NMR- based structure elucidation is most often combined with data from mass spectrometry or chemical information, e.g., monosaccharide composition or methylation analysis.[66] Carbohydrates normally have at least two NMR-active nuclei, ^{13}C and ^{1}H, but also less frequently used nuclei like ^{2}H,[67] ^{3}H, ^{11}B, ^{15}N, ^{17}O, ^{19}F,[68−70] and ^{31}P can be used for studies of natural or synthetic oligosaccharides. The dispersion of resonances in the carbon spectra is favorable, but the amount of material needed to acquire such spectra is relatively high due to the low natural abundance of ^{13}C. However, advances in both hardware and pulse sequences have reduced the amount needed. In practical terms, about 1 mg of a pure trisaccharide is enough to perform a complete structural assignment by both ^{1}H and ^{13}C NMR spectroscopy. When sample amounts are further limited, ^{1}H NMR spectra can be measured down to nanomole quantities. The study of small sample amounts will be discussed in detail in section V. When comparing chemical shift values it is important that the reference data is measured at the same temperature and that the data are based on the same internal reference or one that can be correlated in a simple manner.

1. Number of Sugar Residues

A good starting point for a structural analysis is the anomeric proton chemical shift. Integration of the anomeric resonances offers an initial estimate on the number of different monosaccharide residues present. The anomeric proton resonances are found in the shift range 4.4−5.5 ppm. The remaining ring proton resonances are found in the range 3−4.2 ppm in unprotected oligosaccharides. Additionally, the number of anomeric C1 resonances present in a 1D ^{13}C NMR spectrum will confirm the number of different residues. Such results can also be obtained from 2D $^{13}C-^{1}H$ HSQC,[71] HMQC,[72,73] or HMBC[74] spectra, which in many cases are more sensitive than a 1D ^{13}C spectrum.

2. Constituent Monosaccharides

Homonuclear TOCSY and DQF−COSY spectra are useful in the identification of individual monosaccharide residues. In TOCSY spectra of oligosaccharides acquired with a fairly long mixing time (>100 ms), it is often possible to measure the size of the coupling constants and the correlations to reveal the identity of the residue. In cases with significant

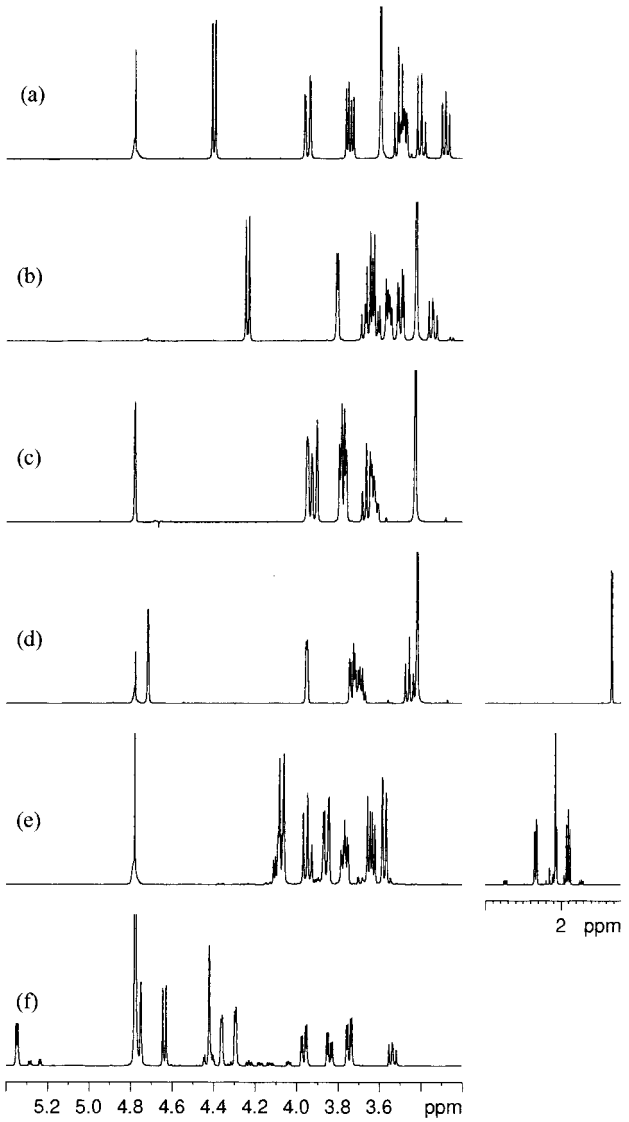
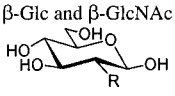
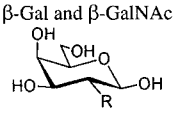
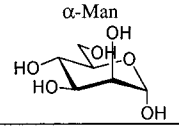
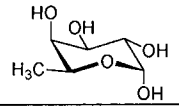
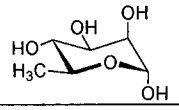
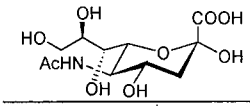
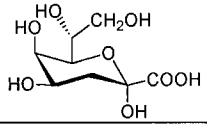
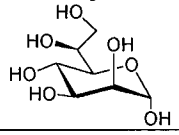
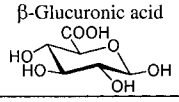
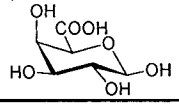


Figure 1. 1D ^{1}H spectra showing the coupling patterns for some of the monosaccharides present in Table 2: (a) Methyl β-D-glucopyranoside; (b) Methyl β-D-galactopyranoside; (c) Methyl α-D-mannopyranoside; (d) Methyl α-L-rhamnoside; (e) *N*-acetylneuraminic acid; (f) D-galacturonic acid. All spectra were acquired on a Varian Unity 500 MHz spectrometer. Spectra were acquired in D_2O at 25 °C, except for c, which was acquired at 35 °C.

overlap in the bulk region (3−4.2 ppm), a 1D TOCSY[24,29] may be useful in resolving ambiguities. In Table 2 the most common sugars are listed with their NMR characteristics and coupling pattern, and NMR spectra of selected monosaccharide residues are shown in Figure 1. Both ^{1}H and ^{13}C chemical shifts for most monosaccharides can be found in the literature (Tables 2 and 3), and based on such values, an assignment of the individual residues can be made. The ^{13}C chemical shift values can easily be obtained from a HSQC or HMQC spectrum. The TOCSY and HSQC (or HMQC) data may also be obtained simultaneously using the 2D version of the HSQC-TOCSY[80−82] or the HMQC-TOCSY[80,83,84] experiments. These experiments are useful and give additional dispersion in the carbon dimension, which may facilitate the assignment of individual spin systems.

Table 2. Selected Sugars[a] and Their Characteristic NMR Features

	Characteristic chemical shifts and J-coupling pattern
β-Glc and β-GlcNAc  R = OH, NHAc	Glc : J_α (Hz) - 3.6, 9.5, 9.5, 9.5 [75] J_β (Hz) - 7.8, 9.5, 9.5, 9.5 [75] The coupling pattern is similar for GlcNAc GlcNAc : δ_{Nac} ~ 2 ppm Upfield shift of $\delta_{C2,\alpha}$ ~ 55.4 ppm, $\delta_{C2,\beta}$ ~ 58 ppm[75,76]
β-Gal and β-GalNAc  R = OH, NHAc	Gal : J_α (Hz) - 3.8, 10, 3.8, 1 [75] J_β (Hz) - 8, 10, 3.8, 1 [75] The coupling pattern is similar for GalNAc GalNAc : δ_{Nac} ~ 2 ppm Upfield shift of $\delta_{C2,\alpha}$ ~ 51.4 ppm, $\delta_{C2,\beta}$ ~ 54.9 ppm[75,76]
α-Man 	J_α (Hz) - 1.8, 3.8, 10.0, 9.8 [75] J_β (Hz) - 1.5, 3.8, 10.0, 9.8 [75]
α-L-Fuc 	δ_{C6} ~ 16.3 ppm[76] and δ_{H6} ~ 1.1 ppm[75] Similar coupling pattern to Gal, J_{56} = 6.3 Hz[75]
α-L-Rha 	δ_{C6} ~ 18.0 ppm[76] and δ_{H6} ~ 1.2 ppm[75] Similar coupling pattern to Man, J_{56} = 6.2 Hz[75]
α-NeuAc 	δ_{H3ax} ~ 1.9 ppm; δ_{H3eq} ~ 2.3 ppm [77] Upfield shift of δ_{C3} $J_{\alpha,\ C1\text{-}H3ax}$ ~ 6 Hz; $J_{\beta,\ C1\text{-}H3ax}$ < 1 Hz [78,79]
α-Kdo[b] 	Upfield shift of C3 ~ 34.8 ppm[76] Coupling pattern similar to NeuAc acid for anomeric configuration.
α-Heptose 	Similar J-coupling pattern to Man For identification assignment of C7 is required, where both a L and D form can be found
β-Glucuronic acid 	Similar coupling pattern to Glc δ_{C6} pH dependent pH = 7.8 $\delta_{\alpha,\ C6}$ =176.9 ppm, $\delta_{\beta,\ C6}$ =177.6 ppm [76] pH = 1.8 $\delta_{\alpha,\ C6}$ =172.9 ppm, $\delta_{\beta,\ C6}$ =173.8 ppm [76]
β-Galacturonic acid 	Similar coupling pattern to Gal δ_{C6} pH dependent pH ~ 6 $\delta_{\alpha,\ C6}$ =172.6 ppm, $\delta_{\beta,\ C6}$ =173.5 ppm [76]

[a] All the sugars listed are D unless otherwise noted. [b] Kdo = 3-deoxy-D-*manno*-octulosonic acid.

Intraresidue NOEs or ROEs are also useful when small coupling constants are present in the spin systems, and a normal TOCSY experiment is difficult to carry out successfully.

For NeuAc or Kdo derivatives (see Table 2) without an anomeric proton, characteristic signals as the $H3_{eq}$ or $H3_{ax}$ protons are a good starting point for the assignments. The methyl groups in terminal deoxy-sugars are equally useful.

3. Anomeric Configuration

Normally the α-anomer resonates downfield compared to the *β*-anomer in D-pyranoses in $^4C_1$ conformation. The vicinal coupling constant between the anomeric H1 and the H2 indicates the relative orientation of the two protons. If they are both in an axial configuration in pyranose structures, a large coupling constant (7−8 Hz) is observed, whereas if they are equatorial−axial, this is smaller ($J_{1,\ 2} \sim 4$ Hz), and for axial−equatorial or equatorial−equatorial oriented protons, even smaller coupling constants are observed (<2 Hz).[85] This principle can be used when assigning the relative orientation of protons in a hexopyranose ring as first demonstrated by Lemieux et al. in 1958.[86] The ^{13}C chemical shift reveals the anomeric configuration in a manner similar to the proton chemical shifts, but most importantly the one bond $^{13}C-^1H$ coupling constants in pyranoses can be used to determine the anomeric configuration unequivocally.[87] For D sugars in the $^4C_1$ conformation,

Table 3. Chemical Shift Table Collections

author	title	year
De Bruyn, A; Anteunis, M.; Verhegge, G.	^{1}H-NMR study of the diglucopyranoses in D_2O	1975[91]
Bock, K.; Thøgersen, H.	Nuclear magnetic resonance spectroscopy in the study of mono- and oligosaccharides	1982[75]
Bock, K.; Pedersen, C.	Carbon-13 nuclear magnetic resonance spectroscopy of monosaccharides	1983[76]
Vliegenthart, J. F. G.; Dorland, L; van Halbeek, H.	High resolution, ^{1}H-nuclear magnetic resonance spectroscopy as a tool in the structural analysis of carbohydrates related to glycoproteins	1983[56]
Bock, K.; Pedersen, C.; Pedersen, H.	Carbon-13 nuclear magnetic resonance data for oligosaccharides.	1984[92]
Shashkov, A. S.; Nifant'ev, N. E.; Amochaeva, V. Y.; Kochetkov, N. K.	^{1}H and ^{13}C NMR data for 2-*O*-,3-*O*- and 2,3-Di-*O*-glycosylated methyl α- and β-D-glucopyranosides and β-D-galactopyranosides	1993[93]
Bock, K.; Duus, J. Ø.	A conformational study of hydroxymethyl groups in carbohydrates investigated by ^{1}H NMR spectroscopy	1994[94]
Hounsell, E. F.	^{1}H NMR in the structural and conformational analysis of oligosaccharides and glycoconjugates	1995[36]
Hobley, P.; Howarth, O.; Ibbett, R. N.	^{1}H and ^{13}C NMR shifts for aldopyranose and aldofuranose monosaccharides: Conformational analysis and solvent dependence	1996[95]
Khatuntseva, E. A.; Shashkov, A. S.; Nifant'ev, N. E.	^{1}H and ^{13}C NMR data for 3-*O*-, 4-*O*-, and 3,4-di-*O*-glycosylated methyl α-L-rhamnopyranosides	1997[96]

a $^1J_{C1,H1} \sim 170$ Hz indicates an α-anomeric sugar configuration whereas $^1J_{C1,H1} \sim 160$ Hz indicates a β-anomeric sugar configuration.[87] This is reversed for L sugars. The use of one-bond coupling constants in furanose structures does not correlate in the same way with the anomeric structure. Several experiments can be used to measure these one-bond coupling constants as discussed below. For sugars such as NeuAc and Kdo with no anomeric proton, the anomeric configuration can be obtained from measurements of the carbonyl−$H3_{eq}$/$H3_{ax}$ coupling constants,[78,79] the C2−H3 coupling constants,[77] or from chemical shifts of the H4 and H6 protons, respectively.

4. Linkage and Sequence

Both the ^{1}H and the ^{13}C chemical shift may give an indication of the linkage type if the chemical shifts for the specific linkage have been reported previously. The effect of glycosylation depends on the linkage type, and the changes in the chemical shift are in general larger at the glycosylation site than at neighboring positions. Interresidue NOEs may give information about the glycosidic linkage, but it should be kept in mind that the strongest NOE might not be between the protons across the glycosidic linkage.[88,89] A HMBC experiment can also give linkage information, keeping in mind that both intra- and interresidue correlations are seen.

5. Position of Appended Groups

The proton and carbon chemical shifts are sensitive to the attachment of a non-carbohydrate group like a methyl, acetyl, sulfate, or a phosphate group. Attachment of such a group will affect the proton and carbon resonances where the group is located. Normally downfield shifts ∼0.2−0.5 ppm are observed[35] for protons and higher $\Delta\delta$ values for ^{13}C. This places these resonances in a less crowded area of the spectra and helps the identification of modified residues. Such appended groups may also contain NMR-active nuclei, which may give rise to additional splittings due to couplings (e.g., ^{31}P−^{1}H long-range couplings). The use of other homo- or heteronuclear correlations may help in the determination of their position.

The approaches outlined in Scheme 1 can help to solve a given structural problem depending on the size of the oligosaccharide studied. As pointed out above, many of the resonances are found in a narrow chemical shift range, and this can make it problematic to distinguish resonances which are close in chemical shift or align signals when comparing different spectra or spectral regions. It is generally necessary to use good software programs to handle the complex data. The program PRONTO (developed at Carlsberg Laboratory by Mogens Kjær[90]) is freely available from the web site http://www.crc.dk/chem/pronto/welcome.html. This package greatly facilitates the assignment and enables the user to analyze several 2D spectra or multidimensional spectra simultaneously using different color codes for each experiment and accurately align spectral regions of particular interest.

6. Classical NMR Methods

Carbohydrate samples are normally dissolved in D_2O, and when working with carbohydrates containing charged groups, like Kdo or phosphates, care should be taken to report the pH of the sample,[97,98] in particular if more samples or structures should be compared. If the structures contain non-carbohydrate moieties, solvent mixtures may be used such as DMSO, methanol, or $CDCl_3$. It can be an advantage to use a mixture of H_2O/D_2O (e.g., 9:1) to be able to observe exchangeable protons as amide H in the NAc group. Spectra are often acquired at close to room temperature, but raising the temperature can give better resolution by sharpening of the resonances, lowering the viscosity of the sample, and increasing the tumbling rate. However, changing the temperature toward the freezing point can be used to optimize the observed NOEs for oligosaccharides, where often small NOEs are observed at room temperature for tri- and tetrasaccharides at 500 MHz. Use of 5% acetone-d_6 as lock signal in D_2O samples can improve the cancellation in difference spectroscopy, due to the much sharper lock signal and less temperature-dependent chemical shift of the acetone signal.

^{13}C chemical shifts are often obtained from multidimensional heteronuclear proton observe experiments using an inverse detection probe, because the

Scheme 1

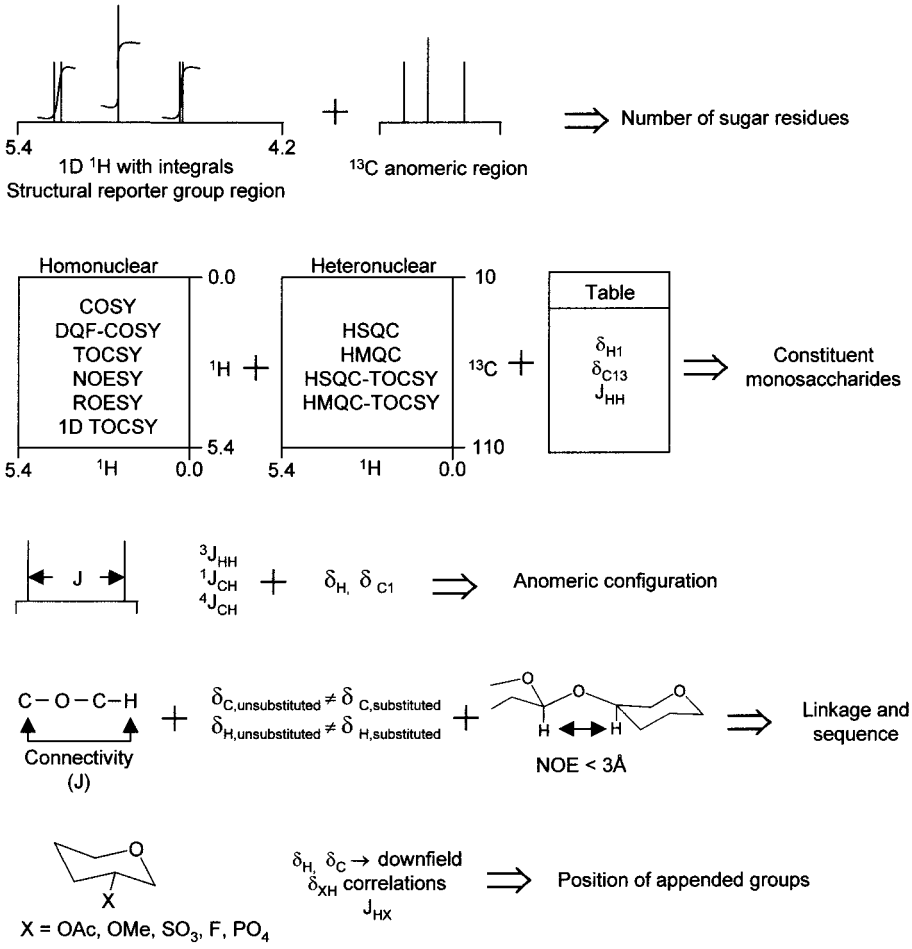


Table 4. NMR Pulse Sequence Abbreviations[a]

experiment		ref
COSY	correlation spectroscopy	113,114
DQF−COSY	double quantum filtered-COSY	115−117
HOHAHA/TOCSY	homonuclear Hartmann Hahn/total correlation spectroscopy	116,118−121
HMBC	heteronuclear multiple bond correlation	74,122−124
HMQC	heteronuclear multiple quantum coherence	72,73,125
HSQC	heteronuclear single quantum coherence	71,126,127
NOESY	nuclear Overhauser enhancement spectroscopy	128,129
ROESY	rotating frame Overhauser enhancement spectroscopy	130,131

[a] Gradient versions of the experiments are normally denoted with a g in front of the experiment name.

1D carbon observe experiment requires more compound. With the introduction of pulse-field gradients, many of the NMR experiments have gained additional sensitivity, because the gradient-enhanced experiments give fewer spectral artifacts, allow for better solvent suppression, and require a shorter phase cycle.[99] In the traditional pulse sequences without gradients, the phase cycling is important because it is used to remove experimental artifacts and to select a specific kind of data. Many 1D, 2D, 3D, and 4D homonuclear and heteronuclear experiments have been adapted to gradient versions.[99] In Table 4 are given the abbreviations and key references to standard pulse sequences.

The most commonly used experiment for the measurement of $^1J_{C,H}$ coupling constants are heteronuclear 2D experiments, although these couplings can also be measured from coupled 1D ^{13}C spectra or from the ^{13}C satellites in ^{1}H NMR spectra.[100] Among the 2D heteronuclear experiments are *J*-resolved spectroscopy[101] as well as the nondecoupled version of the HMQC and HSQC experiments. Several studies have discussed which experiments are best for the study of carbohydrates.[102,103] The $^1J_{C,H}$ couplings can also be measured together with long-range coupling constants using a modified hetero (ω_1) half-filtered TOCSY experiment.[104] Uhrin et al. reported a sensitivity- and gradient-enhanced hetero (ω_1) half-filtered TOCSY experiment which allows a scaling in the F_1 dimension to include or remove the one-bond coupling constants.[105] This scaling allows for an additional splitting which may help in the coupling constant determinations. The coupling constants are obtained from E-COSY-type multiplets,[106−108] and analysis of these gives the long-range heteronuclear coupling constants in the F_2 dimension.

The use of S^3E and S^3CT techniques may also be used in the determination of $^1J_{C,H}$.[109−112]

There are several ways to measure long-range coupling constants in carbohydrates both 2D[132,133] and 1D experiments.[134−137] Many reports in the literature are based on acquiring spectra on samples which are specifically[138−141] or uniformly[142,143] ^{13}C labeled.

The linkage site identification is often done by homonuclear 1D and 2D NOESY or ROESY or the heteronuclear HMBC types of experiments. Recently an application of a Relayed-ROESY experiment has been used to selectively identify a 1→2 glycosidic linkage in polysaccharides.[144] The selection of cross-peaks from 1→2 linkages is achieved by tuning the duration of the TOCSY mixing period. The sensitivity of this technique may be superior to the HMBC on natural abundance samples, although it is dependent on the size of the ROE. Also, the 2D HMQC−NOESY[145] and HSQC−NOESY[80] spectra can give connectivities across the glycosidic linkage and may be helpful in cases with severe overlap due to the separation in the carbon dimension.

B. New NMR Methods

Some of the most promising NMR methods published during the past few years are illustrated below. This will not be a comprehensive list but primarily a selection of methods with new principles or methods with potential application in carbohydrate structural assignment.

In the description of classical NMR methods, the experiments are generally either direct observe 1D or 2D homo- or heteronuclear experiments, such as DQF−COSY, NOESY, and HSQC. Many can be combined to design experiments with multiple evolution times, which after transformation are seen as 3D or higher. The use of high-dimension experiments has been one of the keys to the successful development of protein NMR, where the higher dimensions have allowed the separation of the many closely resonating signals. The rapid development in the field of protein NMR assignment has been dependent on the development of general methods for isotope enrichment of proteins using expression in bacteria or yeast. This is generally not applicable to carbohydrates, but it has been demonstrated in special cases as an interesting approach, which will be described in section IV about polysaccharides. Some examples of 3D experiments using ^{13}C-enriched samples will also be described there.

The methods for carbohydrate NMR assignment, therefore, generally rely on natural abundance samples with mainly ^1H homonuclear 3D experiments.[146−149] The first description of a 3D experiment on a carbohydrate was by Vuister et al.,[149] who demonstrated the usefulness of an NOE-HOHAHA 3D experiment for the assignment of a diantennary asparagine-linked oligosaccharide. The experiment can be seen as a combination of two 2D experiments to reduce the overlap. The same group later published a heteronuclear 3D $^{13}C-^1H$ HMQC−NOESY experiment on the same sample.[145] The main advantage is the increased separation of resonances in the ^{13}C dimension. However, in the same publication the corresponding 2D HMQC−NOESY, which is more widely used, was also described.

Homans and co-workers[146,147] described several 3D experiments for the assignment of complex oligosaccharides. The combination of two 2D techniques into either 3D HOHAHA−COSY or ROESY−COSY[146] has proven to be valuable for the assignment of overlapping signals in spin systems with either gluco- or galacto-configured monosaccharides, respectively. The HOHAHA transfer works well for the gluco configuration with large couplings throughout the spin systems, while transfer through NOE contacts works best in the galacto isomer. In the 3D experiments, planes can be selected which essentially look like 2D COSY spectra for each spin system. In a further extension of the HOHAHA experiment, a pseudo 4D experiment was designed using a selective pulse at the anomeric proton of a given residue to reduce the formally four dimensions into three in an HOHAHA−HOHAHA−COSY experiment.[147] A 3D HOHAHA−HOHAHA experiment from Rutherford and Homans[150] utilized a constant time element in the first incremented dimension, tuned to achieve decoupling of the three-bond coupling between H1 and H2 in β-GlcNAc residues and thereby increase the effective resolution for assignment.

Real 3D experiments have not been used to a large extent in the field of carbohydrate structural assignment, whereas the reduction of multidimensional experiments into 1D or 2D experiments is more common. The main reason is the long time required to obtain 3D or 4D spectra with good digital resolution. However, some examples of 3D spectra have been published either with homonuclear 3D[151] or heteronuclear 3D[143] using a ^{13}C-enriched sample to extract detailed information about the bound conformation of oligosaccharides to lectins.

The implementation and application of Gaussian-shaped pulses to reduce 2D experiments to 1D was first described by Freeman and co-workers in 1984.[152] The key idea is to replace one (or more) of the hard nonselective pulses in a normal 2D experiment, like a TOCSY or COSY, with a soft selective pulse at a given resonance. The resulting 1D spectrum will correspond to the slice in the 2D spectrum at this resonance. The advantages are that many scans at a given resonance can be acquired to give good signal-to-noise and digital resolution. This method is well suited for carbohydrates as the structural reporter groups especially the anomeric protons are ideal for the selective excitation by soft pulses. Bax[153,154] and Kessler[155,156] described 1D versions of all the standard 2D experiments and demonstrated their use on both peptides and oligosaccharides. The methods have been widely applied in carbohydrate NMR spectroscopy, particularly by implementing this approach into the 1D versions of 2D experiments[148,157−161] but also to soft 2D versions of 3D experiments.[148,162,163]

As the advancements of 2D and 3D methods have progressed rapidly, the implementation of such experiments into 1D analogues has followed and Uhrin and co-workers have contributed significantly.[120,161,164−166] These methods are useful, and the

improved version using pulsed field gradients give clean spectra. This has been reviewed by Uhrin in 1997[29] and by Roumestand in 1999,[24] and therefore, only a few examples will be described below.

Many experiments have focused on accurate measurement of long-range $^{13}C-^{1}H$ coupling constants,[81,105,134,135,163,167,168] and the results have mainly been used for conformational studies. The simple 1D experiments, like 1D TOCSY and 1D NOESY, have proven useful for practical structure characterization of carbohydrates,[158,160,166,169] due to the possibility of obtaining high-quality spectra with good resolution in a reasonable amount of time.

The implementation of selective pulses into pulse sequences can be extended quite far, and multiple selective experiments can be performed with the replacement of more than one hard pulse. This is useful to perform multiple transfer steps sequentially to give specific information of, e.g., interresidue NOEs in TOCSY−NOESY[159] or NOESY−TOCSY[164] or even TOCSY−NOESY−TOCSY[164] experiments. A good alternative to the use of multiple selective pulses is the combination of selective pulses and chemical shifts selective filters for 1D versions of 3D experiments.[161] The implementation of pulse field gradients ensures high sensitivity and clean spectra,[29,170] e.g., in 1D NOE experiments[166] and in general in most of the 1D versions of standard 2D experiment or combinations of these.[166]

An interesting extension of 1D TOCSY has been described by Schraml et al.,[171] who acquired a series of 1D TOCSY experiments by a combination of multiple excitation frequencies with phases of component pulses varied according to a Hadamard matrix. Processing and linear combinations of the spectra gave normal 1D TOCSY spectra in a significantly shorter time than by acquiring the spectra one by one.

A powerful tool in carbohydrate analysis is methylation analysis,[66] which provides information about which hydroxyl groups are substituted. Van Halbeek and co-workers investigated oligosaccharides in H_2O at temperatures below 0 °C either by supercooling or addition of acetone-d_6 to prevent freezing.[172] During the studies the authors noticed that the method can be used to identify positions in the monosaccharide residues of oligosaccharides which are glycosidically linked.[172] The aliphatic protons at carbons with OH attached will show couplings to the OH group at low temperature and can be identified by comparison of spectra obtained in D_2O and H_2O using, e.g., 1D TOCSY or by the line broadening. The remaining aliphatic protons, often with sharper signals, will then correspond to positions of the glycosidic linkages or substituted positions. This method requires only small amounts of material compared to the amounts required for a full NMR structural analysis. If this indirect method fails to identify the glycosidic positions due to overlap, the positions bearing OH can be identified in a 2D COSY by the correlation between OH protons and aliphatic protons. Similar experiments can be carried out in DMSO, where the exchange of OH-protons is slow even at room temperature.[173,174]

Another method based on a similar idea has been published by Bendiak,[175,176] who used peracetylation of free hydroxyl groups with ^{13}C-carbonyl-labeled acetic anhydride either fully enriched or only at the carbonyl carbon. The method can be used to separate free hydroxyl positions from positions involved, e.g., in glycosidic linkages. The protons at acetyl-protected positions will show a three-bond $^{13}C-^{1}H$ coupling constant and can be readily identified. This coupling constant has been shown to be in the range from 2.5 to 4.7 Hz and with no interference from the four-bond heteronuclear coupling constants, which are small and buried within the line width. The assignment of an acetylated position can be performed by a comparison of a gCOSY (gradient COSY) and a carbon-decoupled gCOSY experiment. In complex structures, where the protons cannot be identified directly, the advantage of using a ^{13}C label is the increase in sensitivity in HMBC or heteronuclear COSY experiments. Additionally, the protection with acetate increases the chemical shift range where the aliphatic protons are observed and thereby allows for assignment of more complex structures. This approach has been extended by Jones and Bendiak,[177] who devised a set of 3D experiments utilizing separation of closely resonating signals into more dimensions through attached fully ^{13}C-labeled acetate groups. These techniques can reveal the position of the glycosidic linkage but not the sequential positioning of the individual monosaccharide units. The main disadvantage is the requirement of a chemical derivatization, where acetylation of large oligosaccharides is not always easy to carry out to completion.

In 1995 Otter et al.[178,179] demonstrated the potential benefits of using gradient-enhanced versions of COSY (gCOSY) and double-quantum-filtered COSY (gDQFCOSY)[116] for carbohydrates. However, this has not been used extensively in the field of carbohydrates. The advantage of the gCOSY experiment is often only recognized for the fact that this does not need phase cycling, because the coherence selection and suppression of unwanted signals are carried out using the gradients. However, Otter et al. demonstrated that the experiment has superior sensitivity for observing cross-peaks between proton pairs with small coupling constants, like $^{3}J_{H1,H2}$ in β-D-Man residues. However, the ability to observe correlation due to long-range couplings, ^{4}J and ^{5}J, is more useful, where the observation of cross-peaks between proton pairs on each side of the glycosidic linkage could be an important alternative to the use of the $^{13}C-^{1}H$ HMBC experiments in the determination of the linkage between adjacent residues.[179]

A technique described for detecting signals in regions with overlap from signals other than the normal carbohydrate residues has recently been published by Köver et al.[180] using a benzyl-protected oligosaccharide. The problem is well-known in assignment of protected oligosaccharides from synthetic origin, where the CH_2 resonances overlap with the anomeric signals and other downfield-shifted resonances. The method is based on band-selective suppression of unwanted signals prior to standard 2D experiments, as TOCSY or HSQC. The magnetization

transfer within spin systems in the pulse sequence will then give rise to signals for the carbohydrates residues also in the suppressed region. Paulsen and co-workers[181] previously solved this problem by using benzyl protecting groups specifically deuterated at the CH_2 group.

Homonuclear band-selective decoupling with adiabatic pulses[182−184] has proven to be useful for protein experiments, but to our knowledge it has not yet been used in studies of carbohydrates. These experiments apply decoupling to regions of the spectrum during acquisition of 2D or 3D experiments, e.g., in $^{15}N-^{1}H$ HSQC to the H^{α} region. This increases sensitivity and reduces overlap when homonuclear couplings are suppressed. The technique has potential applications also for carbohydrates, which also have signals in fairly well-defined regions, e.g., anomeric protons or H3's of Kdo or NeuAc.

A recent new approach described by Vincent and Zwahlen[185] can potentially complement the HMBC and NOESY experiments for assigning the glycosidic linkages in large polysaccharides. The correlation between the $^{13}C-^{1}H$ pair at the anomeric position and the ^{1}H at the linkage position is done in a new fashion based on the dipole−dipole cross correlation. Cross-correlated relaxation prior to this has been used to obtain conformational information in labeled biomolecules, such as proteins.[186] The method offers better sensitivity than the HMBC experiment for large polysaccharides having a short T_2 relaxation.[185]

C. Computer-Assisted Structural Analysis

With the above-mentioned approaches it is still a time-consuming and tedious task to assign the primary structure of a given oligo- or polysaccharide. Therefore, several new computational methods have been proposed for assistance in the assignment of the primary structure.

Several groups have worked on developing different concepts based on both ^{1}H NMR data [57,58,63−65,85,187−190] and ^{13}C NMR data.[60−65,85,187] A short description of the methodology behind some of the programs is given below.

Vliegenthart and co-workers developed a ^{1}H and ^{13}C NMR database, SUGABASE, which combines CarbBank and Complex Carbohydrate Structure Data (CCSD) with proton and carbon[92] chemical shifts in a search routine.[57,58,189,191] The search is based on the use of ^{1}H chemical shifts from the structural reporter groups.[56] This concept is based on the fact that it is often sufficient to inspect only certain areas of a spectrum to ascertain the primary structure of a common glycoprotein carbohydrate structure. In the structural reporter group approach the crowded region between 3 and 4 ppm is ignored and only the regions between 4−5.6 ppm and 1−3 ppm are inspected. The anomeric protons, methyl protons, protons attached to a carbon atom in the direct vicinity of a linkage position, and protons attached to deoxy carbon atoms are considered relevant structural reporter groups. The chemical shift values are used for a search in SUGABASE, which is available at the web site http://www.boc.chem.uu.nl/sugabase/databases.html. Due to lack of funding, the database is currently not being updated. The same is true for the CarbBank database, which can be found at the following web site http://www.ccrc.uga.edu.[59]

The following information is needed to do a search in SUGABASE: (1) First, one must select one of the databases: N-linked, O-linked, lactose-type, or polysaccharides. (2) Then the accepted deviation in the proton chemical shift, the match percentage, and the proton chemical shifts should be given. Similarly, the version at the web site allows carbon data to be inserted. (3) The output of the search is a list giving the possible structural hits and their chemical shifts, which subsequently must be inspected manually.

The final manual inspection is crucial because coupling constants are not included in the search and such data may rule out structures obtained in the search which do not match the experimental data.

Jansson and Kenne developed the program CASPER (computer-assisted spectrum evaluation of regular polysaccharides).[63−65,85,187,192,193] This program has been developed to perform a structural analysis of both linear and branched oligo- and polysaccharides using ^{1}H and ^{13}C chemical shift data and $^{1}J_{CH}$ or $^{3}J_{HH}$ scalar coupling constants. The program allows both 1D and 2D data to be used and spectra to be simulated. The database with the chemical shifts, different glycosylation shift, and correction sets for sterically strained structures will be more accurate with the increasing number of assigned structural elements included, particularly with the addition of more data from branched molecules. CASPER can be used to extract glycosylation shifts and correction sets from newly assigned structures and incorporate them into the database. The program is described in detail as follows.[63] (1) First input is the sugar composition and linkage composition obtained from methylation or acetylation analysis. Furthermore, the available ^{1}H and ^{13}C chemical shifts and homo-/heteronuclear coupling constants are used as input. (2) From the above data, all possible structures are generated and their ^{1}H and ^{13}C spectra are simulated. The program sorts the results and removes structures incompatible with the input coupling constants. (3) Finally, the simulated spectra are compared with the experimental data, and the structures are ranked according to the lowest average total difference in chemical shifts. These structures can be evaluated by comparison of the structures based on the input NMR data and the simulated spectra.

Kochetkov and co-workers also developed a computer-assisted approach to aid in the primary structural assignment based on ^{13}C NMR data.[60−62] This has been used in the assignment of linear polysaccharides, where the main change in the ^{13}C chemical shifts of each monosaccharide unit is due to glycosylation effects. These effects are largest at the linkage positions with smaller differences at the adjacent carbon atoms.[60,93,96] The glycosylation-induced shifts are taken as the difference between the value found for a given carbon in a reference disaccharide or oligosaccharide and the chemical shift value reported for the corresponding monosaccharide.

The use of multiple data for a given substitution pattern provides additional confidence in the determination of the glycosylation effect. Subsequently, the database has been expanded to include branched polysaccharides.[61,62] The limiting step has been the amount of data available on branched model systems. However, such data are accumulating, and today a convincing analysis of branched polysaccharides is possible, provided the monosaccharide composition is known. The approach involves three steps.[60,62] (1) The generation of all possible structures of a polysaccharide with a given monosaccharide composition. (2) Calculation of the ^{13}C NMR spectra for each of these structures based on the values taken from the database. (3) A search for the best match between the calculated and experimental spectrum.

For the most likely structure a number S is calculated, which is the squared deviation for the chemical shifts of the signals with identical numbers divided by the number of monosaccharide residues in a repeating unit. All possible structures with values of $S < 1.5$ should be evaluated as potential structures for further inspection.

The approaches described above aim to help the primary structural assignment of different oligo- or polysaccharides. A step forward in order to obtain a faster and more efficient way of performing such assignments would be to use the entire proton spectrum in a digital format, applying pattern recognition by artificial intelligence techniques. Artificial neural networks (ANN) have been used for both ^{1}H[194−198] and ^{13}C[199,200] data. Thomsen and Meyer published the first demonstration in the area of carbohydrates in 1989 by recognition of ^{1}H NMR spectra of simple alditols.[194] The extension of this approach to larger structures was published by Meyer et al. on oligosaccharides derived from xyloglucans.[195] In ANN, the neural network is trained on a subset of spectra with known structures, which subsequently can be used in the evaluation of similar structures. This is achieved by using a feed-forward ANN with back-propagation of errors. The ability for ANN to deduce a structure or suggest parts of a structure, which is recognizable in a given spectrum, is dependent on the spectra used in the training set.[194,196] In several studies it has been shown that if spectra with different spectral artifacts or spectra with added noise are included in the training spectra, ANN will perform better.[194−196] It has also been shown that the use of only one selected region of the spectrum, being either the structural reporter group region or the crowded hump region, will be enough to recognize a spectrum of a given structure.[195] Radomski et al.[196] showed that even with a signal-to-noise level between 0.125 and 0.138, the neural network was able to recognize ∼40% of the spectra. This is a good success rate considering that these spectra are too noisy to allow a structural elucidation by visual inspection. This enables the identification of many carbohydrates which could not have been analyzed otherwise, since they may only be available in minute quantities. However, this is only true if the carbohydrates have been characterized before and are present in the training set. Amendolia and co-workers showed that ANN can be used in the recognition of mixtures of alditols and provide a quantitative estimate of the relative concentrations of the components in the mixtures.[198] Valafar and Valafar recently implemented and described such a search engine which can be accessed at http://www.ccrc.uga.edu. Here scientists can submit a spectrum and test if it should be present in one of the databases available.[197]

The methods discussed above are all based on experimental data either used for training of an ANN system or as the basis for empirical rules or database lookup. A new approach which holds significant potential for the future is calculation of the chemical shifts for carbohydrates based on ab initio methods. There has been increased attention on the use of this methodology in small organic molecules and peptides,[201,202] and the theory has been reviewed recently by Helgaker et al.[203] With access to faster computers, investigations of carbohydrates have also been carried out.[204,205] The technique could potentially be an alternative to the above-mentioned methods for structural determination of compounds not previously assigned with the improvement of simulation methods and increasing speed of computers. A prerequisite for the general use of such methods will, however, be the demonstration of accuracy and reliability of the methodology for known structures.

D. NMR Spectroscopy of Mixtures

The applications of libraries of compounds to identify lead compounds in drug discovery has expanded dramatically during the last years.[206−208] The concept is to create a large number of compounds for active screening of a given property, such as specific binding to a receptor, and new techniques have been developed. NMR is a promising possibility using, e.g., SAR by NMR,[209] techniques based on diffusion editing[210,211] or NOE pumping.[212,213] However, only techniques described specifically for carbohydrates will be mentioned here. The idea behind using NMR spectroscopy for the screening of a mixture of carbohydrate structures having a complex spectrum is that the NMR-based technique should select only the compounds in the mixture which bind to the protein of interest.

The first demonstration of this methodology to carbohydrates utilized transfer NOE,[214] which previously has been used to probe the conformation of a single oligosaccharide structure in the bound state. The transfer NOE experiment[215,216] is based on an effect where a small molecule, such as an oligosaccharide, binds to a large protein with the appropriate on and off rates. Under these experimental conditions, cross relaxation is occurring while the molecule is bound to the protein and transferred to the small molecule in solution. Therefore, the NOEs observed for the small molecule become large and negative as opposed to a nonbinding small molecule with small positive NOEs. If a NOESY spectrum is measured on a mixture of oligosaccharides, the specific oligosaccharide binding to the large protein can thus be identified positively. The technique has been demonstrated on a mixture of oligosaccharides bind-

ing to *Aleuria aurantia* agglutinin[214] and more recently for the identification of an E-selectin antagonist.[217] Furthermore, it has recently been demonstrated that the transfer NOE can be combined with a TOCSY to form a 3D TOCSY−trNOESY experiment for the assignment of the active compound in complex mixture.[218]

A limitation of the transfer NOE technique is that it relies on optimal transfer of NOE, which limits its practical application. An improved technique has been proposed by Meyer[219] using saturation-transfer difference spectroscopy. The idea is to saturate a signal from the protein without hitting any of the oligosaccharide signals. The saturation will dissipate fast to the whole protein and subsequently to the small molecule binding to the protein. This technique is more sensitive and flexible than the corresponding transfer NOE experiment. Additionally, the part of the oligosaccharide in closest contact with the protein will experience the strongest saturation transfer and the binding epitope of the ligand can in some instances be identified. The saturation transfer technique has furthermore been demonstrated to work for screening of molecules binding to a protein bound to solid support in magic angle spinning NMR experiments.[220]

NMR spectroscopy is also a powerful technique in the study of complex mixtures of carbohydrates. These can consist of carbohydrates alone or in mixtures with other components. One approach is to measure normal 1D ^{1}H or ^{13}C spectra of mixtures of, e.g., polysaccharides and then with the use of spectra from isolated structures deduce the composition of the mixtures using statistical methods.[221,222] For even more complex mixtures such as blood plasma, information about the content of low molecular weight compounds such as monosaccharides can be obtained using high-field NMR.[223,224] It is advantageous to suppress the signals from the high molecular weight compounds using methods based on relaxation or diffusion.[225] Finally, chromatography with inline NMR detection has been used for the identification of carbohydrate components in complex mixtures.[226−229]

A special application of NMR to complex mixtures of oligosaccharides is the determination of the substrate specificity for carbohydrate hydrolyzing enzymes directly in the NMR tube. This offers detailed information compared to other methods because intermediate products can be identified positively in the mixture.[230−232] This is one of few techniques which immediately determines the stereochemical outcome of the enzymatic hydrolysis, i.e., determines whether the enzymatic hydrolysis occurs via retention or inversion of the anomeric configuration.[233]

III. Large and Unusual Oligosaccharides

Impressive examples of large oligosaccharides assigned by NMR have been published during recent years. The largest structural assignment published using NMR methods is the megaoligosaccharide rhamnogalacturonan II from red wine consisting roughly of 30 monosaccharide residues.[234] This oligosaccharide has been extensively studied by classical chemical carbohydrate methods, such as methylation analysis and partial hydrolysis, but the structure has only been partially assigned. However, recently du Penhoat et al.[234] reinvestigated the structure using 750 MHz NMR and successfully assigned the major part. This was based on standard techniques (^{1}H DQF−COSY, TOCSY, and NOESY and $^{13}C-^{1}H$ gHSQC), which demonstrates the strength of the methodology when used in combination at high field. On the basis of these experiments, the authors were able to limit the number of possible structures to a few and present data relating to the three-dimensional structure. However, due to the size and heterogeneity of the oligosaccharide, the structure could not univocally be fully assigned, i.e., the order of the blocks was shown to be either A−C−B−D or A−D−B−C (see Figure 2). Recently Vidal et al.[235] published a minor correction to the structure, interchanging the assignment of residue B3 and B4.

Similarly, a large oligosaccharide structure has been assigned by Olsthoorn et al.[236] using a combination of NMR at high field and mass spectrometry. This study focuses on the determination of the linkage between the O-chain and the core of the lipopolysaccharide (LPS) from *Salmonella enetrica* serovar *Typhimurium*.[237] The O-chain has previously been studied in detail and similar core structures investigated, but this was the first full assignment of an intact oligosaccharide from an LPS. It was shown that the galactose linking the O-chain to the core has a β configuration and not an α one as observed for the remaining linkages in the O-chain. The sample investigated contains a mixture of the core and structures with increasing length of repeating units of the O-chain attached. The assignment of this complex sample could therefore only be performed successfully by combining the information from mass spectrometry (fast-atom-bombardment MS, electrospray quadrupole MS, and Nano-ES Q-TOF MS) and high-field (750 MHz) ^{1}H and ^{13}C NMR data. The largest structure assigned in the mixture consists of 22 monosaccharide residues (see Figure 3). The assignment of such a large structure has only been possible using the full arsenal of NMR methods but mainly using well established 2D techniques supplemented by 1D versions of TOCSY experiments. The key to the full assignment was the availability of the good reference data from the pure core structure.[237] However, it should be mentioned that assignments of structures of this size or like the rhamnogalacturonan II mentioned above are only possible because they are made up of different monosaccharides.

A special challenge in chemical shift assignment is in oligosaccharides consisting of one type of monosaccharide residues, particularly if only one type of linkage is present. A extreme example has been published by Flugge et al.[238] for cellulose oligomers. The largest structure fully assigned for both ^{1}H and ^{13}C is (β-D-Glc*p*-(1−4)-)$_5$-β-D-Glc*p*-OMe at 750 MHz for ^{1}H. This could be achieved by conversion of the reducing oligosaccharide into the methyl glycoside using protection and deprotection in order to elimi-

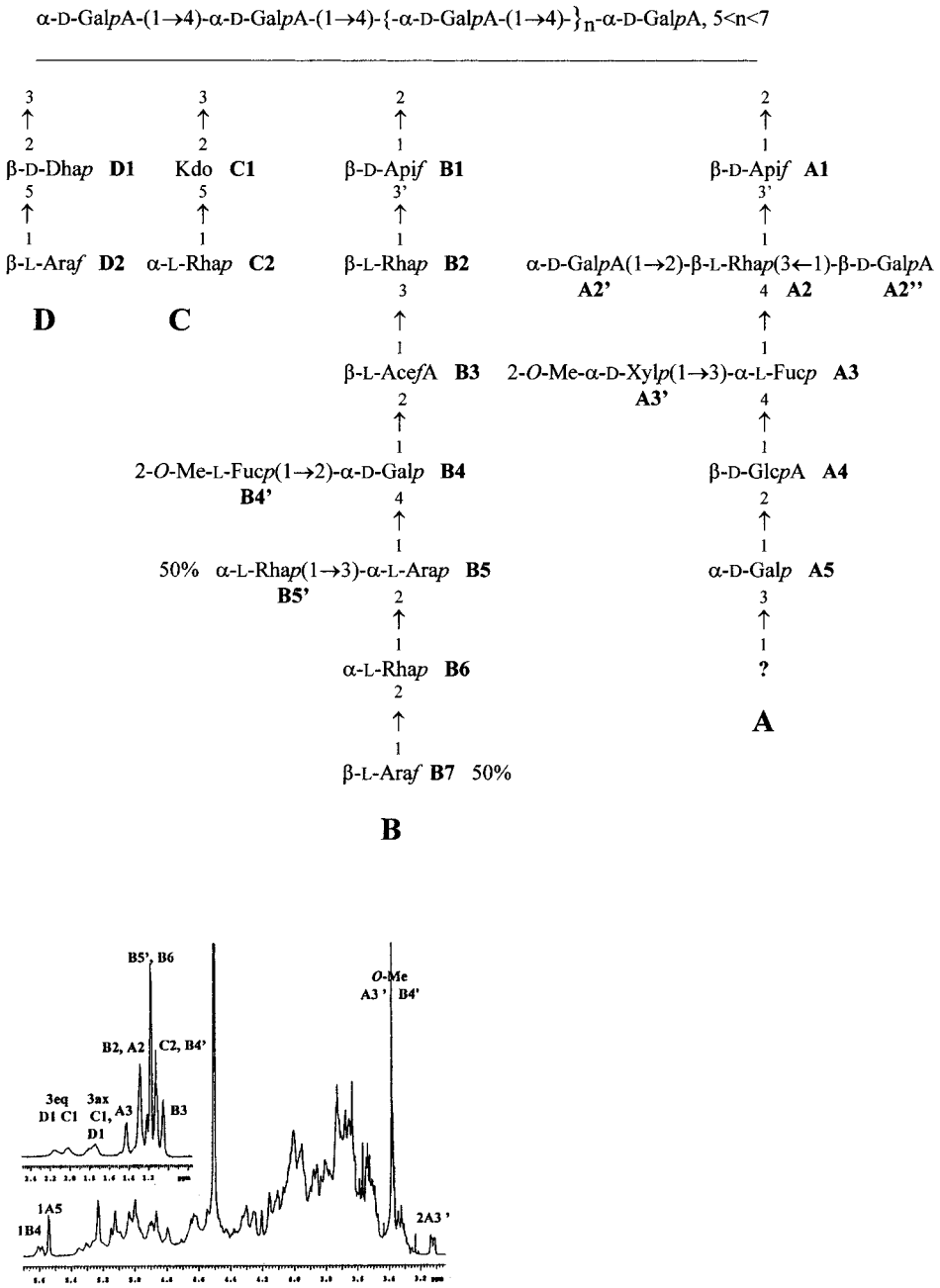


Figure 2. Structure of the "mega-oligosaccharide" rhamnogalacturan II, as determined by NMR spectroscopy by du Penhoat et al. and the 1D ^1H spectrum in D_2O at 40 °C with selected signals labeled. (Reprinted with permission from ref 234. Copyright 1999 Kluwer Academic Publishers.)

nate the anomeric equilibrium. 3D HMQC−TOCSY and HMQC−COSY spectra were used in order to solve the overlap in crowded regions.

Lipopolysaccharides, as discussed above, form a class of compounds which present the widest variety of different monosaccharide residues in their structures both in the O-chain and in the core region.[237,239] Therefore, they offer substantial challenges in their structural determinations. However, NMR spectroscopy has proven to be absolutely essential in the assignment of the particular structures to individual compounds from specific bacterial strains. During the course of such a structural determination of the core region of a species from the *Proteus* strains, a novel type of glycosidic linkage was discovered.[240] This linkage was formed through the acetalic position of a galactosamine residue to the 4- and 6-positions, respectively, of the adjacent galactosamine residue. This linkage has been reported only once before in the literature[241] in a naturally occurring compound as a acetalic linkage to an aromatic residue. The above-mentioned structure is therefore the first time that such a linkage has been reported as an interglycosidic connection. The complete structural assignment of the two types of structures isolated and characterized has been fully determined in two recent publications.[242,243] The characterization of such an unusual type of linkage was only possible by the use of NMR spectroscopy, where the HMBC experiment was used to identify the two long-range connections from H1 of the acetalic carbon to the C6 and C4 protons from the neighboring residue (Figure 4).

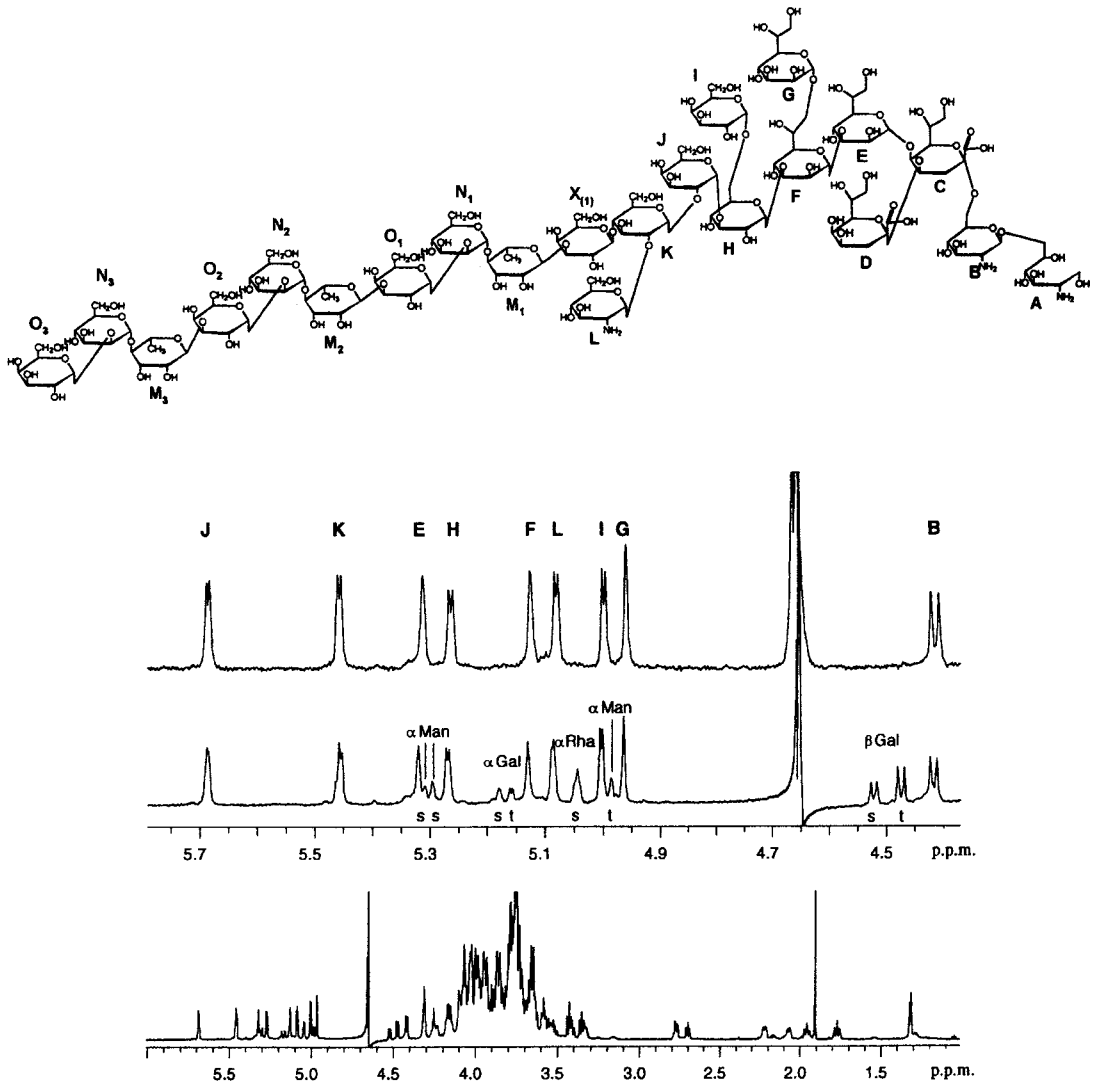


Figure 3. Structure of the largest structure (22 residues) identified of the intact O-chain and core oligosaccharides from the LPS of S*almonella entirica* serovar *Typhimurium* (top). One-dimensional 750 MHz ^{1}H spectra of the LPS sample (lower and middle trace) compared with the spectrum of the core without O-chain (upper trace). (Reprinted with permission of ref 236. Copyright 2000 Blackwell Science.)

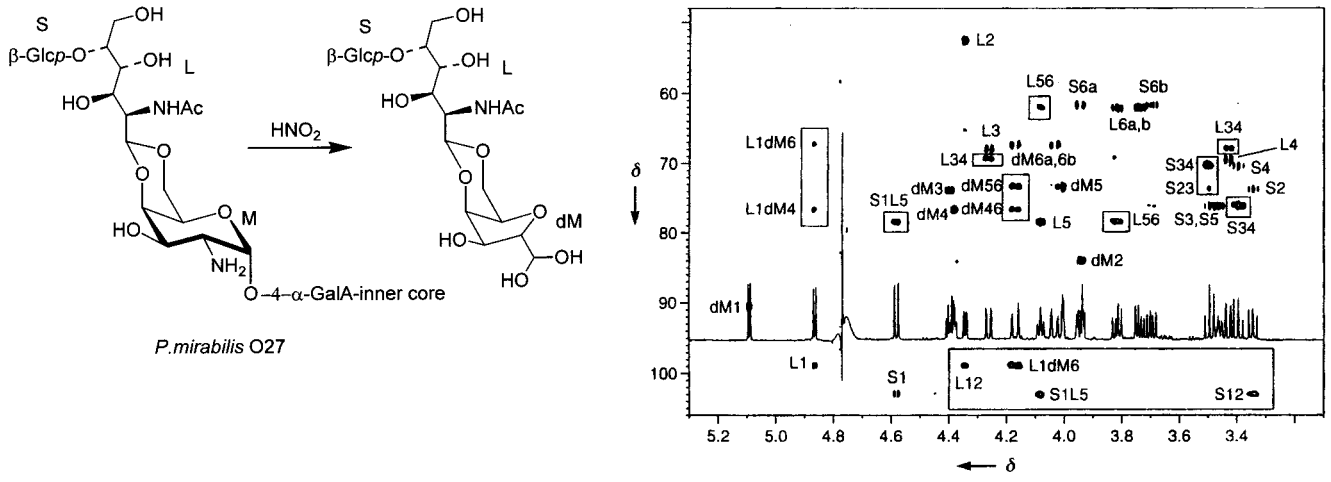


Figure 4. Structure of the open-chain acetal-linked *N*-acetylgalactosamine from the core part of the LPS from *Proteus mirabilis* O27 and the overlay of 1D ^{1}H and 2D HSQC and HMBC spectra of this. (Reprinted with permission from ref 240. Copyright 1999 Wiley-VCH.)

Because several structures have been identified with both substituents at the 4- and 5-position of the open-chain sugar structure, it was easy to rule out the possibility of having a five-membered ring with unusual coupling constants. The relative stereochemistry in the open-chain structure was assigned after

hydrolysis and identification of the coupling pattern of the monosaccharide constituent. The normal methodology used to determine the structural composition of an oligosaccharide is methylation analysis.[66] However, this approach would not have identified the open-chain structures mentioned above with substituents at position 4 or 5 because the results from a methylation analysis would have proposed a 4,6-disubstituted hexoseamine with terminal galactosamine and glucose/galactose residues, respectively. These examples illustrate the power of NMR spectroscopy in structural determination of unknown natural products and also importantly remind scientists to be prepared for unexpected results. The chemical composition of such a type of linkage is not at all unusual because it is one of the often used protecting groups. The acetalic and particularly the 4,6-benzylidene group are similar types of linkages from the acetalic form of an aldehyde or ketone. However, it is surprising that in discussions of the numerous different types of structures in which monosaccharides can be assembled,[6,7] this type of linkage has not been considered even though it at least theoretically would have been a plausible structure. In future discussions of the diversity of structures formed by monosaccharides, this type of linkage has to be included as well and will therefore increase the potential number of structures formed by carbohydrates by another order of magnitude.

IV. Polysaccharides

The field of polysaccharide structure determination has been reviewed by Mulloy in 1996[33] and by Bush and co-workers in 1993[38] and in 1996.[31]

A large number of polysaccharide structures (PS) have been published during the last years, as a result of the growing interest in the relation between biological function and structure of the PS. From a NMR point of view, the main difference between a complex oligosaccharide and a PS is the repeating nature of the PS. Therefore, even large PS can give simple NMR spectra which appear like the spectrum of the repeating oligosaccharide. Because the biosynthesis of natural PS often results in structural variation, the deviation from the perfect repeating structure is the most interesting (or challenging) seen from a structural point of view. The major difference in the NMR spectra of large PS relative to the oligosaccharides is the larger line width, mainly due to short T_2 relaxation. However, also deviation from a perfect repeating structure can contribute to an apparently large line width, which will require more attention in the experimental setup. For example, in phase-sensitive COSY spectra, the large line width can result in partial cancellation and reduced sensitivity. To avoid this, a TOCSY experiment with short mixing time can be used. In experiments using long delays for the transfer of magnetization through small couplings, as in the HMBC experiment, the short T_2 poses a problem. If the T_2 is short, the magnetization is lost during the delays and often low sensitivity is observed. Increased temperature, which lowers the viscosity, will result in a more narrow line width; however, special attention should be paid to labile chemical bonds, e.g., in the presence of uronic acid residues. Alternatively, the average degree of polymerization can be reduced by mechanical stress, like ultrasound treatment.

The structural determination of PS normally involves standard NMR methods combined with chemical methods and MS techniques. However, 1D versions of multidimensional experiments[29,155] are useful in the study of PS. The determination of substitution of the main chain of PS by carbohydrate or non-carbohydrate side chains in less than equimolar amounts is a major problem in the structural elucidation of PS. This results in complex spectra, and often the assignment can be quite difficult. Examples of non-carbohydrate substituents are acetyl (or other acyl groups),[244−250] standard or nonstandard amino acids,[251,252] sulfate,[253,254] and phosphate[255−258] (also cyclic[259] or substituted phosphate[260]). For some substituents special NMR experiments are used for determination of the attachment site. The most widely published example is phosphate, where the NMR-active nuclei ^{31}P can be used directly in ^{31}P−^{1}H correlated experiments[261−264] through the four-bond coupling constants. Alternatively, a comparison of 1D ^{1}H TOCSY with and without ^{31}P decoupling can be used to determine the position of the phosphate group.[265] For other substituents such as acetyl, a useful experiment for assignment of the position of attachment and the exact chemical shift of the acetyl resonances is the HMBC experiment.[175,244,245,266]

In compounds where the substitution occurs in less than equimolar amounts, because the substitution only affects the chemical shifts locally at the residue of attachment, the conclusion will often be that the substitution is present in a statistically random distribution. As an example where this is not the case, Molinaro et al.[244] propose a more complex pattern with stretches of residues with acetylation separated by stretches without and some transition sections for the acetylation of a O-specific caryan PS. These results are based on a combination of high-field NMR and molecular modeling for the interpretation of the chemical shift and NOEs.

Advancements in biotechnology have allowed for efficient production of polysaccharides (and other biomolecules) in bacteria and fungi, where such PS are interesting in connection with the understanding of the biological functions of the microorganisms, especially their pathogenic behavior. This has led to a new approach in the study of PS by NMR using partial or fully ^{13}C enrichment, which has been used extensively for protein 3D structure determination by NMR. In such experiments, ^{13}C-labeled glucose is used as the sole carbon source for the growth of the organism. The application of ^{13}C enrichment has several advantages in the structural determination and can at the same time provide detailed information about the biosynthesis of the PS. The latter was the goal of the first application of this technique, where Jones and co-workers[267,268] assigned the ^{13}C spectrum of the *Klebsiella* K3 serotype PS. The growth condition used both 20% fully ^{13}C-labeled glucose and singly labeled glucose either at C1 or C2, and specifically, the use of ^{13}C COSY spectra has been

described. Using relatively low incorporation of fully labeled glucose can give information about the biosynthetic pathway for the production of a given fragment because two adjacent carbons showing coupling most likely will have their origin from the same glucose residue. The complex biosynthetic pathways often lead to a certain fragment being formed through several routes and therefore can give rise to a pattern of signals from different combinations of labeling (the so-called isotopomer distribution). It is possible to obtain detailed information about the biosynthetic pathways in the organism using ^{2}H-labeled glucose[269] or singly or fully ^{13}C-labeled glucose.[268,270−277] An example of the use of a range of singly ^{13}C-labeled glucose molecules as the carbon source has been published by Kai et al.[277] on branched glucan produced in *Pestalotiopsis*. This gave both the assignment of the ^{13}C chemical shifts and detailed information of the biosynthesis of the glucan polymer.

Prestegard[278] designed 3D experiments for the assignment of all ^{1}H and ^{13}C resonances of ^{13}C-enriched oligosaccharides. Here three dimensions are used to separate resonances overlapping in 2D experiments using both single-quantum and double-quantum chemical shifts to increase separation.[279] The experiment was demonstrated using digalactosyl diacylglyceride but could be useful for larger structures.

Homans described a 3D experiment using uniformly ^{13}C-enriched oligosaccharides in the assignment of exchangeable protons (−OH and −NH) based on HOHAHA−HSQC and NOESY−HSQC (alternatively ROESY−HSQC).[280] These experiments are mainly aimed at the assignment of the indicated protons in conformational studies but could aid in structure determination as well. Homans also described a new enzymatic protocol[281] for specific incorporation of ^{13}C-labeled galactose in the outer part of N-linked glycans on glycoproteins.

The investigation of uniformily ^{13}C-labeled glucan oligosaccharides from *Candida albicans* by Fesik and co-workers[282] by several heteronuclear experiments allowed for a detailed investigation of the specificity of an important glucanosyl transferase involved in the biosynthesis of the cell wall glucan.

Sheng and Cherniak[283] published a study of a glucuronoxylomannan polysaccharide from the pathogenic yeast, *Cryptococus neoformans*, using both 20% and 99% enrichment. This describes the use of 3D HCCH−TOCSY and HCCH−COSY on the fully enriched PS and of gHMBC on the 20% enriched sample. On the basis of these experiments, the repeating sequence was deduced and the advantage of using the ^{13}C−^{13}C coupling for a fully labeled PS was demonstrated for the first time.

Widmalm published several studies on the application of ^{13}C incorporation for the structural elucidation of bacterial PS[276,284] and on development of methods.[285] A key method described is the use of 2D ^{13}C−^{13}C TOCSY experiments with both ^{13}C and ^{1}H detection,[285] based on methods[286] well established for fully ^{13}C-labeled proteins. This circumvents the problem of limited coherence transfer by normal ^{1}H TOCSY in monosaccharide residues with small J couplings, like mannose or galactose. The one-bond ^{13}C−^{13}C coupling constants of about 45 Hz are less dependent on the configuration of the monosaccharides than the three-bond ^{1}H−^{1}H couplings. Due to the large coupling constants, short mixing times (10−20 ms) are required for coherence transfer, which is important for large PS having short T_2. The method[285] was demonstrated using a PS approximately 20% enriched. However, based on a ^{13}C−^{13}C DQF−COSY experiment, it could be proven that the incorporation of fully labeled glucose predominately occurs without disruption of the monosaccharide residues, meaning that even a fairly low incorporation allows transfer through ^{13}C−^{13}C bonds.

Recently, Vinogradov et al.[263] applied ^{13}C enrichment for a detailed structural elucidation of large yeast PS. The study of P*ichia pastoris* mannans also gave the structure of oligosaccharide side chains in the PS, even though present in only a low abundance. This was possible because of the high sensitivity in standard heteronuclear experiments, such as HSQC−TOCSY and HCQC−NOESY for samples being close to 100% ^{13}C-enriched.

Several studies have been carried out on ^{13}C-enriched bacterial PS by Bush and co-workers,[287−289] but this has mainly focused on the conformational and dynamic properties of the PS. Enrichment allows the measurement of extra parameters, like long-range ^{13}C−^{13}C coupling constants and ^{13}C relaxation rates used in the assessment of the conformational preferences of the compounds.[23] Likewise, Homans and co-workers[143] used ^{13}C enrichment in an investigation of the conformation of the oligosaccharide sialyl Lewis^{X} both in solution and bound to E-selectin.

It can be concluded that use of ^{13}C incorporation facilitates structure elucidation for large PS, where it would not have been possible without labeling. Additional information is provided, and a more detailed analysis of complex PS can be carried out.

V. NMR Analysis of Small Sample Amounts

NMR spectroscopy is considered relatively insensitive with respect to the amount of sample needed in order to obtain good-quality structural data. This limitation of NMR compared to other analytical techniques, such as mass spectrometry (MS),[11,16,21] has to be seen in light of the detailed structural information provided by NMR spectroscopy. The amount of sample needed is dependent on the NMR data required. Less compound is required if only ^{1}H NMR data is needed compared to problems where ^{13}C data is required. Even more sample would be required to obtain ^{15}N spectra. Several approaches have been used to increase the sensitivity of NMR, such as an increase in the magnetic field strength, developments in hardware, new developments in probe designs and RF coils,[290] and the developments of new sample tubes. Therefore, it is possible today to study much smaller amounts of sample by NMR spectroscopy than just a few years ago.

Advances in probe and NMR sample tube design and their use in the area of carbohydrates in solution or bound to resins or cells will be discussed below. A

full description of the advances and aspects in the design of small volume probes will not be attempted, but the reader is referred to a recent review by Lacey et al. covering this topic.[290] Examples of non-carbohydrate compounds and glycopeptides are included to prove the usefulness of the approach and to show the new possibilities and opportunities which such probes offer in the study of carbohydrates, oligosaccharides, and mimics of oligosaccharides such as glycopeptides.[291−293]

A. NMR Probes and Sample Tubes

In conventional liquid-state probes, a large volume of the sample is outside the detection area of the RF coil. The reason is that the magnetic field defined by the RF coil needs to be homogeneous in order to obtain sharp and undistorted resonances. This is achieved in conventional NMR tubes by approximating the tube to an infinitely long cylinder with excess sample volume on each side of the observable volume of the receiver coil. This means that for a homogeneous solution state sample, small distortions of the magnetic field are present at the edges of the homogeneous magnetic field. These distortions at the interfaces (liquid/glass/air or liquid/air) are mainly due to differences in the magnetic susceptibility between the sample, the glass, and the surroundings. Minimizing this "excess" sample volume is the most obvious way to increase the sensitivity of NMR on a given sample to concentrate the solute within the receiver coil. Such a decrease in the sample volume can be done by insertion of plugs into the sample tubes at both the top and the bottom, which will lead to a decrease in the liquid volume outside the detection region. Such sample tubes are commercially available for several tube diameters. The plugs should ideally be made of a material in which the magnetic susceptibility is matched to the solvent used in order to minimize the magnetic field distortions at the interface. For a 5-mm sample tube, this can result in a decrease of the sample volume from 600 to 300 μL. A further decrease in the amount of sample needed can be achieved by using a 3-mm or even thinner microprobe or new flow probes using capillaries. The benefits of reduced diameter RF coils was demonstrated many years ago.[294,295]

Probes with a narrower coil diameter and smaller tubes are commercially available for several field strengths. In a 3 mm probe, the sample volume used can be as little as 120 μL and can be further reduced to ~70 μL using inserts or shigemi tubes. Several studies have demonstrated that such probes show an increase in sensitivity.[296−300] Much research has been devoted to new coil design[290,301,302] with some probes having a solenoidal coil design rather than the saddle-shaped RF coil used in conventional liquid-state high-resolution probes. The benefit of a solenoidal coil design is due to its increased sensitivity of approximately 2.6 times over the saddle coil geometry for the same length and diameter of the coil.[303] The need for new probe design to study ever smaller sample amounts to keep up with the other analytical techniques has resulted in an increased interest in coupling different liquid separation techniques directly to NMR using new flow probe designs. Combining high-performance liquid chromatography (HPLC), capillary electrophoresis (CE), and CE chromatography, etc., with NMR has already been demonstrated with sample volumes in the range from 1nL to 100 μL.[229,290] The advances and applications of these techniques has been described in several reviews[227,229,290,304−306] and articles.[307,308]

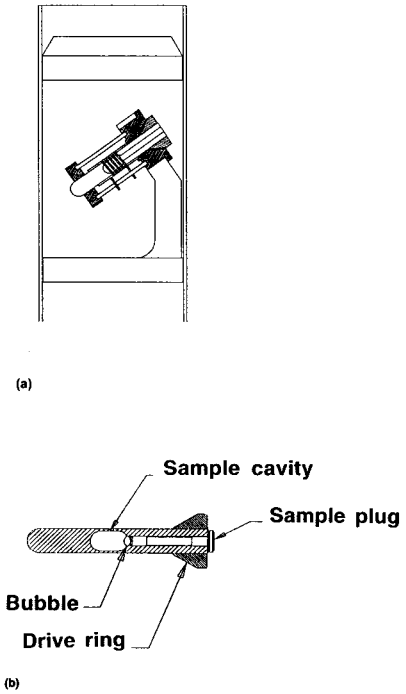


Figure 5. (a) Schematic cross section of a ^{1}H Nano·NMR probe showing the positioning of the sample tube at the magic angle and the solonidal RF coil. (b) Sample tube (Reprinted with permission from ref 337. Copyright 1997 Marcel Dekker.)

Another way to obtain high-resolution spectra and to remove the problem of magnetic susceptibility induced line broadening caused by heterogeneity at the interfaces of the sample is by using magic angle spinning of liquid samples.[309] Spinning with rates above 2 kHz at the magic angle is done by tilting the sample along the magic angle at $\theta = 54.7°$ with respect to the static magnetic field (B_0), the so-called MAS. The NMR company Varian has developed such a high-resolution probe called the Nano·NMR probe. Likewise, the company Bruker has developed a similar HR-MAS probe. Both use the principle of MAS to remove or reduce the increased line width caused by differences in magnetic susceptibility, homonuclear dipolar interactions, and magnetic anisotropy effects.[305,310,311] These effects are reduced to zero because the $(3\cos^2\theta - 1)$ part of the Hamiltonian disappears. The size of the sample cavity for the Varian nanotubes is 40 μL, and with the whole cavity placed in the receiver coil area, the nanoprobe has a high filling factor. A schematic cross section of the nanoprobe and tube can be seen in Figure 5. The maximum volume used is 40 μL, but using as little as 4μL is possible.[305] The Bruker rotors contain 25−80 μL using spacers, which ideally gives a spherical-shaped sample cavity.[311] The spherical shape is known to decrease the magnetic susceptibility induced line broadening. It is crucial to avoid air

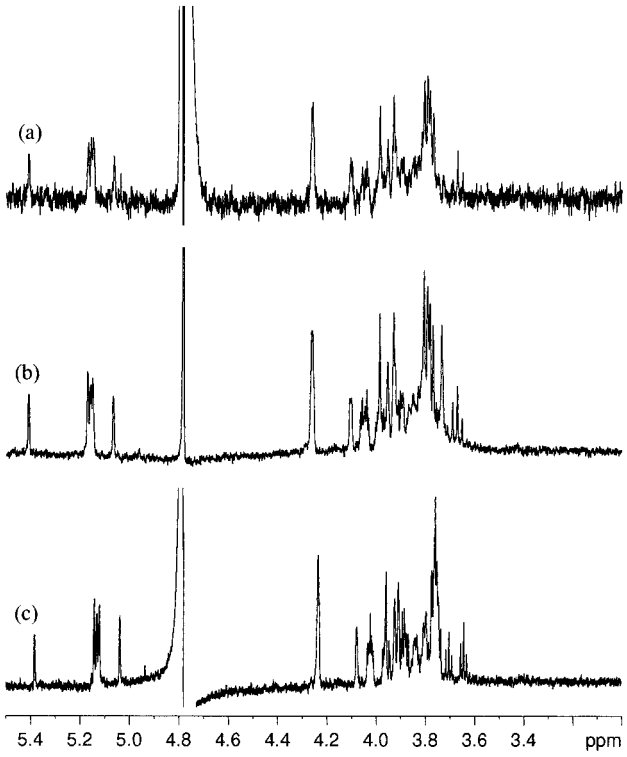


Figure 6. One-dimensional ^{1}H spectra of 5 nmol of the pentamannan α-D- Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→3)-α-D-Man*p*-(1→2)-α-D-Man*p* isolated from *Pichia-(Hansenula) holsti*.[258] The spectra were acquired on the following probes: (a) 5 mm triple-resonance probe at 500 MHz with a sample volume of 600 μL of D_2O; (b) 4 mm ^{1}H-observe Nano·NMR probe at 500 MHz with a sample volume of 40 μL of D_2O; (c) 3 mm triple-resonance probe at 800 MHz with a sample volume of 120 μL of D_2O. All spectra were acquired with 256 scans. The spectra shown have been transformed with a 0.5 Hz line broadening.

bubbles in the sample cell with these rotors. The design of the nanoprobe and the HR-MAS probe is different from most conventional probes because they have a solenoidal RF coil as described above. There are subtle differences in the design and performance of the two probes where a higher spinning rate can be obtained in the HR-MAS probe. The spinning of the sample gives rise to spinning sidebands, which are most obvious for large peaks for which signals may be observed at frequencies corresponding to several times a multiple of the spinning speed. To avoid such effects in the spectra, it is advantageous to spin at a rate which will bring the first spinning sidebands outside the chemical shift range. The required spin rate is dependent on the field strength of the spectrometer, and therefore, operation of a high-field spectrometer requires a high spinning rate to avoid interference with spinning sidebands. Another effect resulting from the high spinning rate is sample heating. This is a well-known phenomenon in solid-state NMR.[312]

Figure 6 illustrates the spectral quality and sensitivity observed using different probes and field strengths with the same amount of solute (5 nmol) in different sample volumes.

In most cases the conventional NMR pulse sequences used in liquid-state NMR can be used on both the nanoprobe and the HR-MAS probe. However, it is reported that better performance is obtained for pulse sequences with continuous spin-lock fields, e.g., TOCSY experiments using adiabatic mixing pulses.[313] These probes may also be equipped with a pulse field gradient coil, with the effective gradient along the magic angle. The use of such gradients and the advantages of spinning at the magic angle have been reported by Maas et al.,[314] who have shown how the use of gradients facilitate the suppression of t_1-noise, especially in heteronuclear experiments, and minimize extra spinning sidebands in MAS homonuclear correlation experiments.

The advantages of using small sample amounts spinning at the magic angle is not just the reduction in liquid volume compared to conventional probes. The shorter 90° pulse on the observe channel may be taken as a number indicating that such probes have a high sensitivity (Figure 6).[303] However, these probes have other applications and can be used to study heterogeneous samples.[304−306,311,315] This includes studies of heterogeneous samples originating from plant materials,[53,316,317] seeds,[318]and cell specimen.[319−321] Another area where this is most useful is in the analysis of resin-bound compounds from solid-phase synthesis.[310,315,322−324]

The new instrumental advances using a decreased sample size to obtain a higher concentration and sensitivity for a given amount has one drawback which may be relevant to some carbohydrate-containing samples. With the increase in concentration it is not always possible to decrease the sample volume due to either aggregation or precipitation at the high concentrations. In such cases, there is no immediate benefit in going to a smaller sample volume but it is more advisable to choose the most sensitive probe, which is normally the probe with the largest sample volume within the receiver coil.[290]

The recent introduction of cryogenic NMR probes will be useful in studies of sample-limited problems with a reported increase in sensitivity by a factor of 3−4.[227,325,326] This increase in sensitivity is achieved by cooling the radio frequency coils below ca. 25 K and eventually the preamplifier, which will reduce the thermal noise and increase the probe quality factor (Q).[325,327−329] However, the increased sensitivity is dependent on the conductivity of the sample, as studies have shown that for a high salt concentration the sensitivity gain is decreased. Flynn et al. recently reported that this may be overcome by encapsulating, e.g., a protein in a reverse micelle solution.[330] The advances encountered with this probe will enable the throughput in screening to be increased.[331,332] Several applications of the cryoprobe have appeared in the literature[326,333,334] but to our knowledge none so far in the field of carbohydrate chemistry.

B. Examples of Structural Analysis with Small Sample Amounts

Several examples using 2.5 and 3 mm microprobes in studies of carbohydrates have been reported. Using a 2.5 mm microprobe, Ruud et al.[335] were able to obtain the first complete structure of a major lipopolysaccharide (LPS) molecular species in *Chlamydia trachomatis* using 110 μg of sample by hetero-

and homonuclear assignments. It was shown that the structure of the deacetylated LPS was α-Kdo-(2→8)-α-Kdo-(2→4)-α-Kdo-(2→6)-β-D-Glc*p*N−(1→6)-α-D-Glc*p*N-1,4′-bisphosphate.

The nanoprobe as well as the HR-MAS probe have been used in several studies of minute solution- state samples of oligosaccharides. Manzi et al. showed the use of a nanoprobe for the structural elucidation of a glycosaminoglycan (GAG) chain isolated from human melanoma cells in culture.[336,337] Both 1D and 2D NMR homonuclear spectra were obtained on samples containing between 10 and 25 μg of a fragment varying in size from 1 to 5 monosaccharide residues linked to a methylumbelliferyl (MU) residue. Although not all resonance assignments could be obtained, the structural data enabled the determination of the unusual terminal α-GalNAc residue in the sequence α-D-GalNAc*p*-(1→4)-β-D-GlcA*p*-(1→3)-β-D-Gal*p*-(1→3)-β-D-Gal*p*-(1→4)-β-D-Xyl*p*-MU. Since the initial report of this structure with a terminal αGalNAc, an enzyme able to transfer such a residue to a growing GAG chain has been found confirming the structural characterization.[337,338]

These initial studies were based on measuring ^{1}H NMR data. Later studies of oligosaccharides have shown the requirements for larger sample amounts to obtain heteronuclear correlated spectra. Gilbert et al. showed that in order to acquire a gradient HMBC, which is the least sensitive of the normally used heteronuclear correlation experiments, a 5 mM (200 nmol) solution of a tetrasaccharide linked to 6-(5-fluorescein-carboxamido)hexanoic acid succimidyl ester was needed.[339] The HMBC spectrum was acquired in 18.5 h, and the concentration in the nanotube was 15 times higher than what could be achieved in a normal 5-mm sample tube. The spectrum enabled verification of the structure by detecting the $^3J_{C,H}$ correlations across the glycosidic bonds.

The isolation and separation of oligosaccharides of similar sizes and composition often necessitates the use of several purification steps. Depending on the analytical method, it can be advantageous to analyze the fractions directly from an analytical column as in HPLC NMR and LC NMR.[290,305] These techniques do, however, require complex setups for both probe and the separation technique, and the nanoprobe may be a good alternative to the flow probes. Broberg et al. performed a structural elucidation on fractions that were 3−18 μg of oligosaccharides ranging from a tetra- to heptasaccharide.[340] The arabinoxylan oligosaccharides were separated using a high-performance anion exchange chromatography-PAD system, and the samples were isolated directly from an analytical column, desalted, and analyzed in the nanoprobe. Both 1D and 2D homonuclear experiments were acquired, and 3 μg (4 nmol) was sufficient to obtain useful data for the structural characterization of a pentasaccharide containing four β-D-xylopyranosyl groups and one α-L-arabinofuranosyl group as shown in Figure 7. The chemical shift values and the connectivities in the ROESY spectrum gave the sequential linkage pattern between the sugar monomers. Both the DQF−COSY, TOCSY, and ROESY experiments on the 3-μg (4 nmol) sample were acquired in less than 24 h with a 500 MHz instrument. Evaluation of the ROESY spectrum in Figure 7c shows that this sample amount is close to the detection limit for a molecule of this size with current instrumentation. This is also about the same amount of sample needed on a single bead in order to do a full structural characterization of an octapeptide using homonuclear DQF−COSY and NOESY spectra acquired on a nanoprobe.[341]

All of the above-mentioned studies using the MAS high-resolution probes might have been acquired using a conventional NMR probe and an extensive amount of time at a higher field spectrometer. However, this is not the case for the studies described in the following where carbohydrates from plant cells or resin-bound molecules are being studied. In these heterogeneous samples, several different NMR techniques not conventionally used may be useful. One way of obtaining better spectra of samples, which contain both small molecules as well as larger rigid components, is by using the difference in spin−spin relaxation times (T_2). Protons present in large rigid components have shorter T_2 times than the protons present in the small molecules. The Carr−Purcell−Meiboom−Gill (CPMG) sequence[342] may be used as a T_2 filter removing the broad signals originating from lipids, solidlike materials, or polymer beads scaffold. Broberg et al. have, among others, shown that using this method it is possible to quantify the amount of a certain metabolite present in a plant sample.[317] Care must be taken when setting up the experiment since the value for the τ delay in the spin−echo element, the loop counter, and the relation between the duration of the spin−echo sequence and the rotor spinning rate have an effect on the appearance of the spectra.[317] Looking at red alga in the HR-MAS probe, Broberg et al. also demonstrated that in-situ identification of the major metabolites is possible.[317] These studies include both 1D and 2D homo- and heteronuclear experiments on alga samples containing between 1.5 and 1.6 mg (dry weight) estimated to contain amounts 30−60 μg of the main metabolite floridoside.[316]

In many cases it is only possible to study the soluble core part of LPS using a conventional NMR probe,[335] but using high-resolution MAS NMR opens the possibility to study intact LPS molecules and LPS directly present on the cell surfaces as shown by Jachymek et al.[343] and Czaja et al.[344] Jachymek et al. reports a full structural NMR study based on data from an LPS suspension isolated from *Yokenella regensburgei* bacteria. With 0.5−1 mg of cells (dry weight estimate) in 30 μL of D_2O it was possible to identify the O-polysaccharide structure especially in cases where the reference structure was already known. An important result is that the structure of the LPS may be the same whether studied on the bacterial surface, in an LPS suspension, or as the isolated polysaccharide. The fact that the structural reporter group signals can be seen enables the direct identification of a surface-linked polysaccharide, particularly in cases where a reference compound from a prior identification is available.[222]

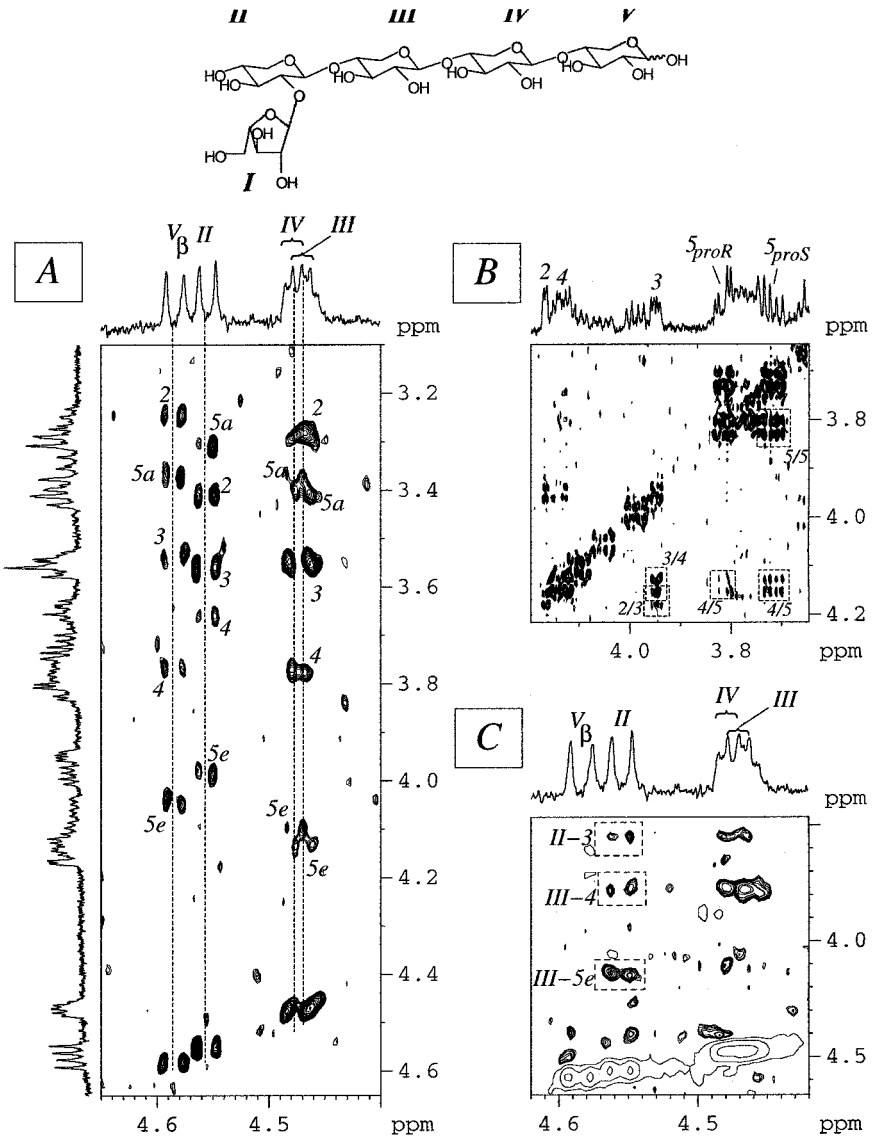


Figure 7. Sections from 2D ^{1}H TOCSY (A), DQF−COSY (B), and ROESY (C) spectra of approximately 4 nmol of a five-residue arabinoxylan sample measured at 500 MHz using a 4 mm ^{1}H-observe Nano·NMR probe. (Reprinted with permission from ref 340. Copyright 2000 Elsevier.)

Many oligosaccharides or glycopeptides of interest cannot be isolated from natural sources and therefore have to be synthesized. The increased interest in oligosaccharides and the fact that they are difficult and labor intensive to synthesize has initiated research into the area of solid-phase synthesis of oligosaccharides.[207,345] The advances in solid-phase synthesis of oligosaccharides are not as advanced as the solid-phase synthesis of peptides and oligonucleotides. The development of solid-phase oligosaccharide synthesis and particularly the monitoring of each reaction step is an area where high-resolution magic angle spinning NMR can be used. Seeberger et al.[324,345] demonstrated this approach using HR-MAS NMR in the analysis of a resin-bound trimeric oligosaccharide synthesized on a polystyrene resin support. Using 20 mg of swelled resin in the sample tube, it was possible to follow the reactions and obtain proton, carbon, and HMQC spectra within a reasonable time of about 2 h per carbon experiment. Using ^{13}C-enriched acetyl protection group for the synthesis of sialyl Lewis^{X} on solid support, Wong and co-workers[346] also followed the progress of the synthesis with conventional ^{13}C gated decoupling spectra.

Studies have shown that glycopeptides can function as mimics of carbohydrates,[291−293,347] giving valuable information about the nature of the interaction between a protein and carbohydrate. The synthesis of glycopeptides can be accomplished as solid-phase library synthesis with the aim of producing a library of glycopeptides which can be screened for biological activity on the solid-phase beads. After performing the biological screening on the bead, it is also desirable to do the structural analysis of the compounds attached to the resin to avoid possible chemical modifications or degradation of the compound during the cleavage reaction.

The spectral quality achievable on a nanoprobe in a structural analysis of a resin-bound glycopeptide will be illustrated below. The glycopeptide was synthesized in its acetyl-protected form at the Carlsberg Laboratory by Dr. Morten Meldal and Dr. Koen M. Halkes by a published method.[348] The 1D ^{1}H NMR

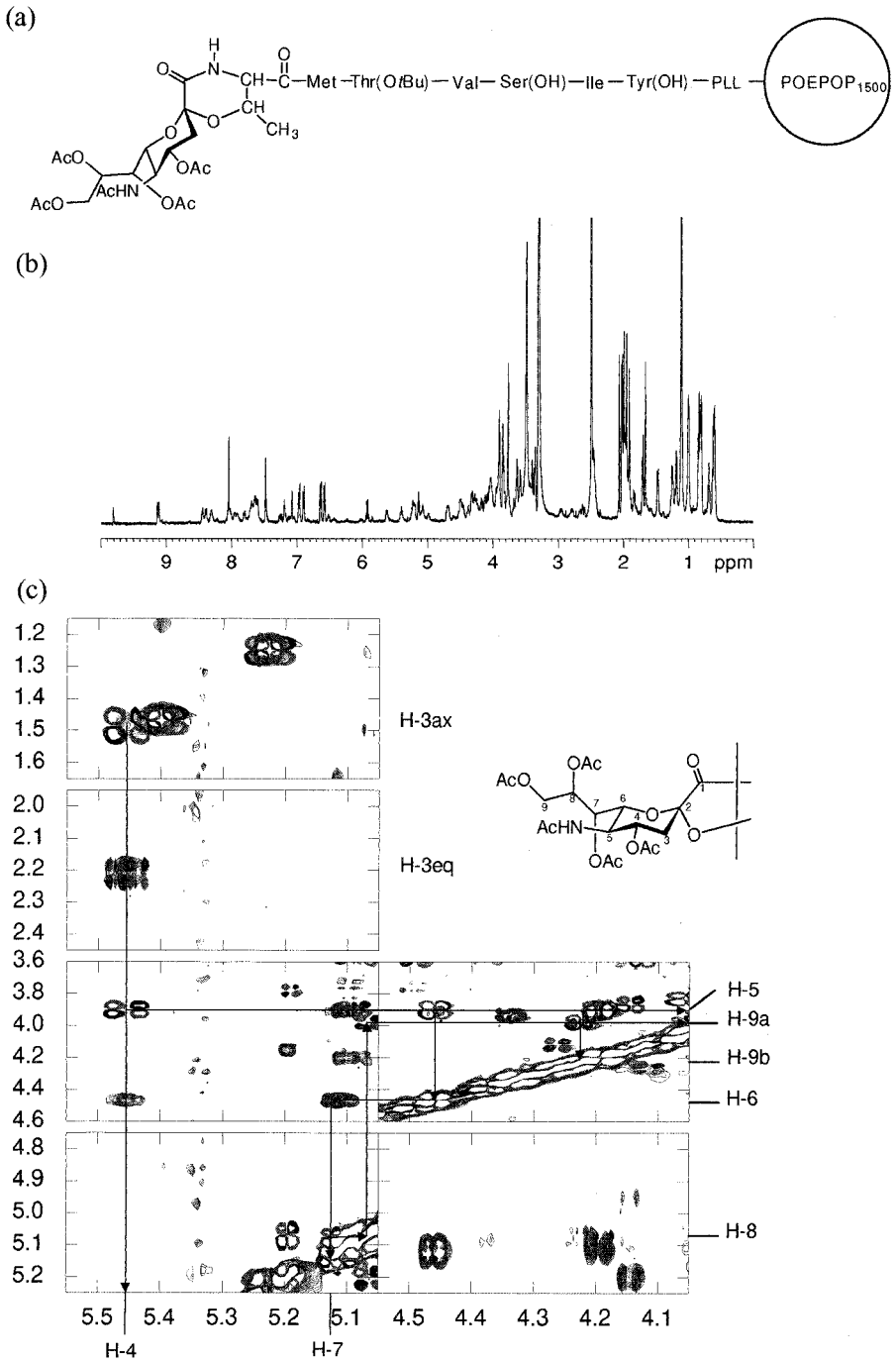


Figure 8. (a) Structure of the glycopeptide. (b) 1D ^{1}H spectrum acquired on 5−10 beads with 64 scans. (c) Full ^{1}H assignments of the acetyl-protected sialic acid with the assignments indicated, shown is a NOESY and DQF−COSY spectrum. The bottom spectra show an application of the PRONTO program.[90] All spectra were acquired on a Varian Unity 500 MHz spectrometer equipped with a 4 mm ^{1}H-observe Nano·NMR probe. The spectra were obtained at 25 °C in DMSO-d_6.

spectrum shown in Figure 8b was acquired on a sample containing 5−10 POEPOP[349] beads in DMSO-d_6. The full structural characterization was performed using both 1D and 2D homonuclear DQF−COSY and NOESY experiments. The 1D spectrum in Figure 8b shows that good high-resolution spectra can be obtained for both the peptide and the carbohydrate part without disturbance from the polymer support and with a spectral quality comparable with that obtained on a normal liquid-state sample. The quality of spectra obtained may be very different depending on the solid support used in the study as these have been shown to have a significant effect on the spectral quality.[350,351] As seen in Figure 8a, the sialic acid forms a closed lactam ring with the threonine. The lactam ring was proven both by NMR and MALDI-TOF mass spectrometry. The assignment of the acetyl-protected sialic acid is shown in Figure 8c, where DQF−COSY and NOESY spectra are overlaid and the assignment of all the sialic protons are shown. This example describes how efficiently nanoprobe NMR may enable the study of glycopeptides and other resin-bound compounds from library synthesis.

VI. Conclusions

The previous discussion and all the above-mentioned results clearly substantiate the importance of NMR spectroscopy in the structural elucidation of carbohydrates and their derivatives. Even though many of the examples presented are specialties for the experienced expert, NMR spectroscopy in structural characterization either of synthetic or isolated samples will continue to be one of the most important techniques used in the daily laboratory work with carbohydrates. NMR spectroscopy is today probably the most often used analytical technique which can provide invaluable information about carbohydrate molecules. It is therefore also expected that the advances in NMR spectroscopy will be followed intensely by carbohydrate scientist to take advantage of new developments or experiments published in the field. In particular, work on new probe design will most likely result in continued increasing sensitivity and with the demand for the amount of analytical sample going well below the microgram scale and approaching the high picomole range.

A few weeks before the submission of this review, the first data obtained at 900 MHz were made public. This was achieved in a venture between Varian NMR systems and Oxford Instruments, and other companies are expected to follow soon. Because the magnet technology is pushed to the limit, the magnet is designed as a "pumped" system which allows the superconducting material available today to carry a higher current and withstand the higher field to produce the homogeneous fields required in high-resolution NMR spectroscopy. Several papers have addressed the possibility of reaching 1 GHz NMR instruments or above,[352−354] and it is still an area which receives much attention in physics.

However, with the current magnet technology, it is more likely that developmental work will be devoted to new probe designs such as cryoprobes,[326] capillary flow probes, automated use of flow probes,[355] or nanoprobes/high-resolution MAS probes as described above. It was shown here that the actual sensitivity obtained in a nanoprobe operating at 500 MHz was similar to the sensitivity obtained in a 3-mm probe on an 800 MHz instrument, off course without the same spectral dispersion. Therefore, for many oligosaccharide samples it is more advantageous to use the new probe technology and the many new pulse sequences designed to sample specific properties than going to higher field strength with the associated substantially much higher expense both in investment and running cost.

VII. Acknowledgments

Several of the high-field examples were measured on the 750/800 MHz instrument at the Danish National NMR Instrument Center for studies of biological macromolecules. C.H.G. has been supported by grant no. 9900687 from The Danish Technical Research Council. K.B. thanks The Ib Henriksens Foundation for support.

VIII. References

(1) Dwek, R. A. *Chem. Rev.* **1996**, *96*, 683−720.
(2) Rudd, P. M.; Dwek, R. A. *Crit. Rev. Biochem. Mol. Biol.* **1997**, *32*, 1−100.
(3) Rudd, P. M.; Endo, T.; Colominas, C.; Groth, D.; Wheeler, S. F.; Harvey, D. J.; Wormald, M. R.; Serban, H.; Prusiner, S. B.; Kobata, A.; Dwek, R. A. *Proc. Nat. Acad. Sci. U.S.A.* **1999**, *96*, 13044−13049.
(4) Duus, J. Ø.; St. Hilaire, P. M.; Meldal, M.; Bock, K. *Pure Appl. Chem.* **1999**, *71*, 755−765.
(5) Rüdiger, H.; Siebert, H.-C.; Solis, D.; Jimenez-Barbero, J.; Romero, A.; von der Lieth, C.-W.; Diaz-Maurino, T.; Gabius, H.-J. *Curr. Med. Chem.* **2000**, *7*, 389−416.
(6) Laine, R. A. *Pure Appl. Chem.* **1997**, *69*, 1867−1873.
(7) Gabius, H.-J. *Naturwissenschaften* **2000**, *87*, 108−121.
(8) Apweiler, R.; Hermjakob, H.; Sharon, N. *Biochim. Biophys. Acta* **1999**, *1473*, 4−8.
(9) Anumula, K. R.; Dhume, S. T. *Glycobiology* **1998**, *8*, 685−694.
(10) Rassi, Z. E.; Mechref, Y. S. *Capillary Electrophoresis, Theory and Practice*; CRC Press: London, 1997; Chapter 7, pp 273−363.
(11) van der Hoeven, R. A. M.; Hofte, A. J. P.; Tjaden, U. R.; van der Greef, J.; Torto, N.; Gorton, L.; Marko-Varga, G.; Bruggink, C. *Rapid Commun. Mass Spectrom.* **1998**, *12*, 69−74.
(12) Tseng, K.; Hedrick, J. L.; Lebrilla, C. B. *Anal. Chem.* **1999**, *71*, 3747−3754.
(13) Makino, Y.; Omichi, K.; Hase, S. *Anal. Biochem.* **1998**, *264*, 172−179.
(14) Carlwood, J.; Birrell, H.; Organ, A.; Camilleri, P. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 716−723.
(15) Steinberg, P.; Fox, A. *Anal. Chem.* **1999**, *71*, 1914−1917.
(16) Ahn, Y. H.; Yoo, J. S. *Rapid Commun. Mass Spectrom.* **1999**, *13*, 1985−1990.
(17) Venkataraman, G.; Shriver, Z.; Raman, R.; Sasisekharan, R. *Science* **1999**, *286*, 537−542.
(18) Geyer, H.; Geyer, R. *Acta Anat.* **1998**, *161*, 18−35.
(19) Rudd, P. M.; Guile, G. R.; Küster, B.; Harvey, D. J.; Opdenakker, G.; Dwek, R. A. *Nature* **1997**, *388*, 205−207.
(20) Rudd, P. M.; Mattu, T. S.; Zitzmann, N.; Mehta, A.; Colominas, C.; Hart, E.; Opdenakker, G.; Dwek, R. A. *Biotechnol. Genet. Eng. Rev.* **1999**, *16*, 1−21.
(21) Thoru, Y. *Trends Glycosci. Glycotechnol.* **1999**, *11*, 227−232.
(22) Sato, Y.; Suzuki, M.; Nirasawa, T.; Suzuki, A.; Endo, T. *Anal. Chem.* **2000**, *72*, 1207−1216.
(23) Bush, C. A.; Martin-Pastor, M.; Imberty, A. *Annu. Rev. Biophys. Biomol. Struct.* **1999**, *28*, 269−293.
(24) Roumestand, C.; Delay, C.; Gavin, J. A.; Canet, D. *Magn. Reson. Chem.* **1999**, *37*, 451−478.
(25) Poveda, A.; Jimenez-Barbero, J. *Chem. Soc. Rev.* **1998**, *27*, 133−143.
(26) Schneider, H.-J.; Hacket, F.; Rüdiger, V.; Ikeda, H. *Chem. Rev.* **1998**, *98*, 1755−1785.
(27) Widmalm, G. *Carbohydrate Chemistry*; Blackie Academic & Professional: London, 1998; Chapter 11, pp 448−502.
(28) Hounsell, E. F.; Bailey, D. *Glycopeptides and Related Compounds*; Marcel Dekker: New York, 1997; Chapter 13, pp 631−660.
(29) Uhrin, D. *Methods for Structure Elucidation by High-Resolution NMR*; Elsevier: Amsterdam, 1997; Chapter 3, pp 51−89.
(30) Agrawal, P. K.; Pathak, A. K. *Phytochem. Anal.* **1996**, *7*, 113−130.
(31) Bush, C. A. *Encyclopedia of Nuclear Magnetic Resonance*; John Wiley & Sons Ltd: Chichester, 1996; pp 3746−3750.
(32) Lerner, L. E. *NMR Spectroscopy and its Application to Biomedical Research*; Elsevier: Amsterdam, 1996; Chapter 7, pp 313−344.
(33) Mulloy, B. *Mol. Biotechnol.* **1996**, *6*, 241−265.
(34) Peters, T.; Pinto, B. M. *Curr. Opin. Struct. Biol.* **1996**, *6*, 710−720.
(35) van Halbeek, H. *Encyclopedia of Nuclear Magnetic Resonance*; John Wiley & Sons Ltd: Chichester, 1996; pp 1107−1137.
(36) Hounsell, E. F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1995**, *27*, 445−474.
(37) van Halbeek, H. *Curr. Opin. Struct. Biol.* **1994**, *4*, 697−709.
(38) Abeygunawardana, C.; Bush, C. A. *Adv. Biophys. Chem.* **1993**, *3*, 199−249.
(39) Agrawal, P. K. *Phytochemistry* **1992**, *31*, 3307−3330.
(40) Bush, C. A.; Cagas, P. *Adv. Biophys. Chem.* **1992**, *2*, 149−180.
(41) Tjandra, N.; Bax, A. *Science* **1997**, *278*, 1111−1114.
(42) Bolon, P. J.; Prestegard, J. H. *J. Am. Chem. Soc.* **1998**, *120*, 9366−9367.
(43) Kiddle, G. R.; Homans, S. W. *FEBS Lett.* **1998**, *436*, 128−130.
(44) Martin-Pastor, M.; Bush, C. A. *Biochemistry* **2000**, *39*, 4674−4683.
(45) Martin-Pastor, M.; Bush, C. A. *Carbohydr. Res.* **2000**, *323*, 147−155.

(46) Landersjö, C.; Höög, C.; Maliniak, A.; Widmalm, G. *J. Phys. Chem. B* **2000**, *104*, 5618−5624.
(47) Dais, P. *Adv. Carbohydr. Chem. Biochem.* **1995**, *51*, 63−131.
(48) Kjellberg, A.; Widmalm, G. *Biopolymers* **1999**, *50*, 391−399.
(49) Söderman, P.; Widmalm, G. *Magn. Reson. Chem.* **1999**, *37*, 586−590.
(50) Rundlöf, T.; Venable, R. M.; Pastor, R. W.; Kowalewski, J.; Widmalm, G. *J. Am. Chem. Soc.* **1999**, *121*, 11847−11854.
(51) van Duynhoven, J. P. M.; Kulik, A. S.; Jonker, H. R. A.; Haverkamp, J. *Carbohydr. Polym.* **1999**, *40*, 211−219.
(52) Gidley, M. J. *Developments in Carbohydrate Chemistry*; American Association of Cereal Chemists: St. Pauls, MN, 1992; pp 163−191.
(53) Renard, C. M. G. C.; Jarvis, M. C. *Plant Physiol.* **1999**, *119*, 1315−1322.
(54) Zhang, P.; Kylmachyov, A. N.; Brown, S.; Ellington, J. G.; Grandinetti, P. J. *Solid State Nucl. Magn. Reson.* **1998**, *12*, 221−225.
(55) Cheetham, N. W. H.; Tao, L. *Carbohydr. Polym.* **1998**, *36*, 285−292.
(56) Vliegenthart, J. F. G.; Dorland, L.; van Halbeek, H. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 209−374.
(57) van Kuik, J. A.; Vliegenthart, J. F. G. *Trends Glycosci. Glycotechnol.* **1991**, *3*, 229−230.
(58) van Kuik, J. A.; Hård, K.; Vliegenthart, J. F. G. *Carbohydr. Res.* **1992**, *235*, 53−68.
(59) Doubet, S.; Bock, K.; Smith, D.; Darvill, A.; Albersheim, P. *Trends Biochem. Sci.* **1989**, *14*, 475−477.
(60) Lipkind, G. M.; Shashkov, A. S.; Knirel, Y. A.; Vinogradov, E. V.; Kochetkov, N. K. *Carbohydr. Res.* **1988**, *175*, 59−75.
(61) Lipkind, G. M.; Shashkov, A. S.; Kochetkov, N. K. *Carbohydr. Res.* **1990**, *198*, 399−402.
(62) Lipkind, G. M.; Shashkov, A. S.; Nifant'ev, N. E.; Kochetkov, N. K. *Carbohydr. Res.* **1992**, *237*, 11−22.
(63) Jansson, P.-E.; Kenne, L.; Widmalm, G. *J. Chem. Inf. Comput. Sci.* **1991**, *31*, 508−516.
(64) Jansson, P.-E.; Kenne, L.; Widmalm, G. *Anal. Biochem.* **1991**, *199*, 11−17.
(65) Hermansson, K.; Jansson, P.-E.; Kenne, L.; Widmalm, G.; Lindh, F. *Carbohydr. Res.* **1992**, *235*, 69−81.
(66) Lindberg, B.; Lönngren, J. *Methods Enzymol.* **1978**, *50*, 3−33.
(67) Bose-Basu, B.; Zajicek, J.; Bondo, G.; Zhao, S.; Kubsch, M.; Carmichael, I.; Serianni, A. S. *J. Magn. Reson.* **2000**, *144*, 207−216.
(68) Penglis, A. A. E. *Adv. Carbohydr. Chem. Biochem.* **1981**, *38*, 195−285.
(69) Csuk, R.; Glänzer, B. I. *Adv. Carbohydr. Chem. Biochem.* **1988**, *46*, 73−177.
(70) Michalik, M.; Hein, M.; Frank, M. *Carbohydr. Res.* **2000**, *327*, 185−218.
(71) Bodenhausen, G.; Ruben, D. J. *Chem. Phys. Lett.* **1980**, *69*, 185−189.
(72) Müller, L. *J. Am. Chem. Soc.* **1979**, *101*, 4481−4484.
(73) Bax, A.; Griffey, R. H.; Hawkins, B. L. *J. Magn. Reson.* **1983**, *55*, 301−315.
(74) Bax, A.; Summers, M. F. *J. Am. Chem. Soc.* **1986**, *108*, 2093−2094.
(75) Bock, K.; Thøgersen, H. *Annu. Rep. NMR Spectrosc.* **1982**, *13*, 1−57.
(76) Bock, K.; Pedersen, C. *Adv. Carbohydr. Chem. Biochem.* **1983**, *41*, 27−66.
(77) Prytulla, S.; Lambert, J.; Lauterwein, J.; Klessinger, M.; Thiem, J. *Magn. Reson. Chem.* **1990**, *28*, 888−901.
(78) Haverkamp, J.; Spoormaker, T.; Dorland, L.; Vliegenthart, J. F. G.; Schauer, R. *J. Am. Chem. Soc.* **1979**, *101*, 4851−4853.
(79) Prytulla, S.; Lauterwein, J.; Klessinger, M.; Thiem, J. *Carbohydr. Res.* **1991**, *215*, 345−349.
(80) Norwood, T. J.; Boyd, J.; Heritage, J. E.; Soffe, N.; Campbell, I. D. *J. Magn. Reson.* **1990**, *87*, 488−501.
(81) Köver, K. E.; Hruby, V. J.; Uhrin, D. *J. Magn. Reson.* **1997**, *129*, 125−129.
(82) de Beer, T.; van Zuylen, C. W. E. M.; Hård, K.; Boelens, R.; Kaptein, R.; Kamerling, J. P.; Vliegenthart, J. F. G. *FEBS Lett.* **1994**, *348*, 1−6.
(83) Lerner, L.; Bax, A. *J. Magn. Reson.* **1986**, *69*, 375−380.
(84) Cavanagh, J.; Palmer, A. G., III; Wright, P. E.; Rance, M. *J. Magn. Reson.* **1991**, *91*, 429−436.
(85) Jansson, P.-E.; Kenne, L.; Widmalm, G. *Carbohydr. Res.* **1987**, *168*, 67−77.
(86) Lemieux, R. U.; Kullnig, R. K.; Bernstein, H. J.; Schneider, W. G. *J. Am. Chem. Soc.* **1958**, *80*, 6098−6105.
(87) Bock, K.; Pedersen, C. *J. Chem. Soc., Perkin Trans. 2* **1974**, 293−297.
(88) Lemieux, R. U.; Bock, K.; Delbaere, L. T. J.; Koto, S.; Rao, V. S. *Can. J. Chem.* **1980**, *58*, 631−653.
(89) Vinogradov, E. V.; Petersen, B. O.; Thomas-Oates, J. E.; Duus, J. Ø.; Brade, H.; Holst, O. *J. Biol. Chem.* **1998**, *273*, 28122−28131.
(90) Kjær, M.; Andersen, K. V.; Poulsen, F. M. *Methods Enzymol.* **1994**, *239*, 288−307.
(91) De Bruyn, A.; Anteunis, M.; Verhegge, G. *Bull. Soc. Chim. Belg.* **1975**, *84*, 721−734.
(92) Bock, K.; Pedersen, C.; Pedersen, H. *Adv. Carbohydr. Chem. Biochem.* **1984**, *42*, 193−225.
(93) Shashkov, A. S.; Nifant'ev, N. E.; Amochaeva, V. Y.; Kochetkov, N. K. *Magn. Reson. Chem.* **1993**, *31*, 599−605.
(94) Bock, K.; Duus, J. Ø. *J. Carbohydr. Chem.* **1994**, *13*, 513−543.
(95) Hobley, P.; Howarth, O.; Ibbett, R. N. *Magn. Reson. Chem.* **1996**, *34*, 755−760.
(96) Khatuntseva, E. A.; Shashkov, A. S.; Nifant'ev, N. E. *Magn. Reson. Chem.* **1997**, *35*, 414−419.
(97) Jones, C.; Mulloy, B. *Methods in Molecular Biology; Spectroscopic Methods and Analyses*; Humana Press Inc.: Totowa, NJ, 1993; Chapter 6, pp 149−167.
(98) van Halbeek, H. *Methods in Molecular Biology; Spectroscopic Methods and Analyses*; Humana Press Inc.: Totowa, NJ, 1993; Chapter 5, pp 115−148.
(99) Keeler, J.; Clowes, R. T.; Davis, A. L.; Laue, E. D. *Methods Enzymol.* **1994**, *239*, 145−207.
(100) Bock, K.; Pedersen, C. *Carbohydr. Res.* **1985**, *145*, 135−140.
(101) Hall, L. D.; Morris, G. A. *Carbohydr. Res.* **1980**, *82*, 175−184.
(102) Uhrinova, S.; Uhrin, D.; Liptaj, T.; Bella, J.; Hirsch, J. *Magn. Reson. Chem.* **1991**, *29*, 912−922.
(103) Helander, A.; Kenne, L. *Carbohydr. Res.* **1991**, *221*, 245−251.
(104) Kurz, M.; Schmieder, P.; Kessler, H. *Angew. Chem., Int. Ed. Engl.* **1991**, *30*, 1329−1331.
(105) Uhrin, D.; Batta, G.; Hruby, V. J.; Barlow, P. N.; Köver, K. E. *J. Magn. Reson.* **1998**, *130*, 155−161.
(106) Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1985**, *107*, 6394−6396.
(107) Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Chem. Phys.* **1986**, *85*, 6837−6852.
(108) Griesinger, C.; Sørensen, O. W.; Ernst, R. R. *J. Magn. Reson.* **1987**, *75*, 474−492.
(109) Sørensen, M. D.; Meissner, A.; Sørensen, O. W. *J. Biomol. NMR* **1997**, *10*, 181−186.
(110) Meissner, A.; Duus, J. Ø.; Sørensen, O. W. *J. Magn. Reson.* **1997**, *128*, 92−97.
(111) Meissner, A.; Duus, J. Ø.; Sørensen, O. W. *J. Biomol. NMR* **1997**, *10*, 89−94.
(112) Lerche, M. H.; Meissner, A.; Poulsen, F. M.; Sørensen, O. W. *J. Magn. Reson.* **1999**, *140*, 259−263.
(113) Aue, W. P.; Bartholdi, E.; Ernst, R. R. *J. Chem. Phys.* **1976**, *64*, 2229−2246.
(114) Marion, D.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1983**, *113*, 967−974.
(115) Piantini, U.; Sørensen, O. W.; Ernst, R. R. *J. Am. Chem. Soc.* **1982**, *104*, 6800−6801.
(116) Hurd, R. E. *J. Magn. Reson.* **1990**, *87*, 422−428.
(117) Davis, A. L.; Laue, E. D.; Keeler, J.; Moskau, D.; Lohman, J. *J. Magn. Reson.* **1991**, *94*, 637−644.
(118) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *65*, 355−360.
(119) Braunschweiler, L.; Ernst, R. R. *J. Magn. Reson.* **1983**, *53*, 521−528.
(120) Köver, K. E.; Uhrin, D.; Hruby, V. J. *J. Magn. Reson.* **1998**, *130*, 162−168.
(121) Kupce, E.; Schmidt, P.; Rance, M.; Wagner, G. *J. Magn. Reson.* **1998**, *135*, 361−367.
(122) Willker, W.; Leibfritz, D.; Kerssebaum, R.; Bermel, W. *Magn. Reson. Chem.* **1993**, *31*, 287−292.
(123) Ruiz-Cabello, J.; Vuister, G. W.; Moonen, C. T. W.; van Gelderen, P.; Cohen, J. S.; van Zijl, P. C. M. *J. Magn. Reson.* **1992**, *100*, 282−302.
(124) Meissner, A.; Moskau, D.; Nielsen, N. C.; Sørensen, O. W. *J. Magn. Reson.* **1997**, *124*, 245−249.
(125) Hurd, R. E.; John, B. K. *J. Magn. Reson.* **1991**, *91*, 648−653.
(126) Palmer, A. G., III; Cavanagh, J.; Wright, P. E.; Rance, M. *J. Magn. Reson.* **1991**, *93*, 151−170.
(127) Kay, L. E.; Keifer, P.; Saarinen, T. *J. Am. Chem. Soc.* **1992**, *114*, 10663−10665.
(128) Jeener, J.; Meier, B. H.; Bachmann, P.; Ernst, R. R. *J. Chem. Phys.* **1979**, *71*, 4546−4553.
(129) Kumar, A.; Ernst, R. R.; Wüthrich, K. *Biochem. Biophys. Res. Commun.* **1980**, *95*, 1−6.
(130) Bothner-By, A. A.; Stephens, R. L.; Lee, J.-M.; Warren, C. D.; Jeanloz, R. W. *J. Am. Chem. Soc.* **1984**, *106*, 811−813.
(131) Bax, A.; Davis, D. G. *J. Magn. Reson.* **1985**, *63*, 207−213.
(132) Sheng, S.; van Halbeek, H. *J. Magn. Reson.* **1998**, *130*, 296−299.
(133) Uhrin, D.; Varma, V.; Brisson, J.-R. *J. Magn. Reson, Ser. A* **1996**, *119*, 120−124.
(134) Poppe, L.; van Halbeek, H. *J. Magn. Reson.* **1991**, *92*, 636−641.
(135) Poppe, L.; van Halbeek, H. *J. Magn. Reson.* **1991**, *93*, 214−217.
(136) Uhrin, D.; Mele, A.; Boyd, J.; Wormald, M. R.; Dwek, R. A. *J. Magn. Reson.* **1992**, *97*, 411−418.
(137) Uhrin, D.; Mele, A.; Köver, K. E.; Boyd, J.; Dwek, R. A. *J. Magn. Reson, Ser. A* **1994**, *108*, 160−170.

(138) Zhao, S.; Bondo, G.; Zajicek, J.; Serianni, A. S. *Carbohydr. Res.* **1998**, *309*, 145−152.
(139) Bose, B.; Zhao, S.; Stenutz, R.; Cloran, F.; Bondo, P. B.; Bondo, G.; Hertz, B.; Carmichael, I.; Serianni, A. S. *J. Am. Chem. Soc.* **1998**, *120*, 11158−11173.
(140) Church, T. J.; Carmichael, I.; Serianni, A. S. *J. Am. Chem. Soc.* **1997**, *119*, 8946−8964.
(141) Podlasek, C. A.; Stripe, W. A.; Carmichael, I.; Shang, M.; Basu, B.; Serianni, A. S. *J. Am. Chem. Soc.* **1996**, *118*, 1413−1425.
(142) Gitti, R.; Long, G.; Bush, C. A. *Biopolymers* **1994**, *34*, 1327−1338.
(143) Harris, R.; Kiddle, G. R.; Field, R. A.; Milton, M. J.; Ernst, B.; Magnani, J. L.; Homans, S. W. *J. Am. Chem. Soc.* **1999**, *121*, 2546−2551.
(144) Cipollo, J. F.; Trimble, R. B.; Rance, M.; Cavanagh, J. *Anal. Biochem.* **2000**, *278*, 52−58.
(145) de Waard, P.; Boelens, R.; Vuister, G. W.; Vliegenthart, J. F. G. *J. Am. Chem. Soc.* **1990**, *112*, 3232−3234.
(146) Homans, S. W. *Glycobiology* **1992**, *2*, 153−159.
(147) Rutherford, T. J.; Homans, S. W. *Glycobiology* **1992**, *2*, 293−298.
(148) Schröder, H.; Haslinger, E. *Liebigs Ann. Chem.* **1993**, 959−965.
(149) Vuister, G. W.; de Waard, P.; Boelens, R.; Vliegenthart, J. F. G.; Kaptein, R. *J. Am. Chem. Soc.* **1989**, *111*, 772−774.
(150) Rutherford, T. J.; Homans, S. W. *J. Magn. Reson, Ser. B* **1995**, *106*, 10−13.
(151) Scheffler, K.; Brisson, J.-R.; Weisemann, R.; Magnani, J. L.; Wong, W. T.; Ernst, B.; Peters, T. *J. Biomol. NMR* **1997**, *9*, 423−436.
(152) Bauer, C.; Freeman, R.; Frenkiel, T.; Keeler, J.; Shaka, A. J. *J. Magn. Reson.* **1984**, *58*, 442−457.
(153) Davis, D. G.; Bax, A. *J. Am. Chem. Soc.* **1985**, *107*, 7197−7198.
(154) Inagaki, F.; Shimada, I.; Kohda, D.; Suzuki, A.; Bax, A. *J. Magn. Reson.* **1989**, *81*, 186−190.
(155) Kessler, H.; Oschkinat, H.; Griesinger, C. *J. Magn. Reson.* **1986**, *70*, 106−133.
(156) Kessler, H.; Anders, U.; Gemmecker, G.; Steuernagel, S. *J. Magn. Reson.* **1989**, *85*, 1−14.
(157) Bazzo, R.; Edge, C. J.; Dwek, R. A.; Rademacher, T. W. *J. Magn. Reson.* **1990**, *86*, 199−203.
(158) Bricher, H. R.; Müller, C.; Bigler, P. *J. Magn. Reson.* **1990**, *89*, 146−152.
(159) Poppe, L.; van Halbeek, H. *J. Magn. Reson.* **1992**, *96*, 185−190.
(160) Sabesan, S.; Duus, J. Ø.; Fukunaga, T.; Bock, K.; Ludvigsen, S. *J. Am. Chem. Soc.* **1991**, *113*, 3236−3246.
(161) Uhrin, D.; Brisson, J.-R.; Bundle, D. R. *J. Biomol. NMR* **1993**, *3*, 367−373.
(162) Bircher, H.; Müller, C.; Bigler, P. *Magn. Reson. Chem.* **1991**, *29*, 726−729.
(163) Hricovíni, M.; Tvaroska, I.; Uhrin, D.; Batta, G. Y. *J. Carbohydr. Chem.* **1989**, *8*, 389−394.
(164) Uhrin, D.; Brisson, J.-R.; Kogan, G.; Jennings, H. J. *J. Magn. Reson, Ser. B* **1994**, *104*, 289−293.
(165) Uhrin, D.; Brisson, J.-R.; MacLean, L. L.; Richards, J. C.; Perry, M. B. *J. Biomol. NMR* **1994**, *4*, 615−630.
(166) Uhrin, D.; Barlow, P. N. *J. Magn. Reson.* **1997**, *126*, 248−255.
(167) Poppe, L.; York, W. S.; van Halbeek, H. *J. Biomol. NMR* **1993**, *3*, 81−89.
(168) Poppe, L.; Sheng, S.; van Halbeek, H. *J. Magn. Reson, Ser. A* **1994**, *111*, 104−107.
(169) Stott, K.; Keeler, J.; Van, Q. N.; Shaka, A. J. *J. Magn. Reson.* **1997**, *125*, 302−324.
(170) Parella, T. *Magn. Reson. Chem.* **1996**, *34*, 329−347.
(171) Schraml, J.; van Halbeek, H.; De Bruyn, A.; Contreras, R.; Maras, M.; Herdewijn, P. *Magn. Reson. Chem.* **1997**, *35*, 883−888.
(172) Sheng, S.; Cherniak, R.; van Halbeek, H. *Anal. Biochem.* **1998**, *256*, 63−66.
(173) Bock, K.; Lemieux, R. U. *Carbohydr. Res.* **1982**, *100*, 63−74.
(174) Bernet, B.; Vasella, A. *Helv. Chim. Acta* **2000**, *83*, 995−1021.
(175) Bendiak, B. *Carbohydr. Res.* **1999**, *315*, 206−221.
(176) Bendiak, B. *Carbohydr. Res.* **1999**, *321*, 139−139.
(177) Jones, D. N. M.; Bendiak, B. *J. Biomol. NMR* **1999**, *15*, 157−168.
(178) Otter, A.; Hindsgaul, O.; Bundle, D. R. *Carbohydr. Res.* **1995**, *275*, 381−389.
(179) Otter, A.; Bundle, D. R. *J. Magn. Reson, Ser. B* **1995**, *109*, 194−201.
(180) Köver, K. E.; Feher, K.; Szilagyi, L.; Borbas, A.; Herczegh, P.; Liptak, A. *Tetrahedron Lett.* **2000**, *41*, 393−396.
(181) Paulsen, H.; Röben, W.; Heiker, F. R. *Tetrahedron Lett.* **1980**, *21*, 3679−3680.
(182) Kupce, E.; Wagner, G. *J. Magn. Reson, Ser. B* **1996**, *110*, 309−312.
(183) Matsuo, H.; Kupce, E.; Li, H.; Wagner, G. *J. Magn. Reson, Ser. B* **1996**, *111*, 194−198.
(184) Kupce, E.; Wagner, G. *J. Magn. Reson, Ser. B* **1995**, *109*, 329−333.
(185) Vincent, S. J. F.; Zwahlen, C. *J. Am. Chem. Soc.* **2000**, *122*, 8307−8308.
(186) Reif, B.; Hennig, M.; Griesinger, C. *Science* **1997**, *276*, 1230−1233.
(187) Jansson, P.-E.; Kenne, L.; Widmalm, G. *Carbohydr. Res.* **1989**, *188*, 169−191.
(188) Hounsell, E. F.; Wright, D. J. *Carbohydr. Res.* **1990**, *205*, 19−29.
(189) Bot, D. S. M.; Cleij, P.; van 'T Klooster, H. A.; van Halbeek, H.; Veldink, G. A.; Vliegenthart, J. F. G. *J. Chemom.* **1988**, *2*, 11−27.
(190) Griffiths, L. *Magn. Reson. Chem.* **2000**, *38*, 444−451.
(191) Leeflang, B. R.; Faber, E. J.; Erbel, P.; Vliegenthart, J. F. G. *J. Biotechnol.* **2000**, *77*, 115−122.
(192) Stenutz, R.; Erbing, B.; Widmalm, G.; Jansson, P.-E.; Nimmich, W. *Carbohydr. Res.* **1997**, *302*, 79−84.
(193) Stenutz, R.; Jansson, P.-E.; Widmalm, G. *Carbohydr. Res.* **1998**, *306*, 11−17.
(194) Thomsen, J. U.; Meyer, B. *J. Magn. Reson.* **1989**, *84*, 212−217.
(195) Meyer, B.; Hansen, T.; Nute, D.; Albersheim, P.; Darvill, A.; York, W.; Sellers, J. *Science* **1991**, *251*, 542−544.
(196) Radomski, J. P.; van Halbeek, H.; Meyer, B. *Nat. Struct. Biol.* **1994**, *1*, 217−219.
(197) Valafar, F.; Valafar, H. *Trends Anal. Chem.* **1999**, *18*, 508−512.
(198) Amendolia, S. R.; Doppiu, A.; Ganadu, M. L.; Lubinu, G. *Anal. Chem.* **1998**, *70*, 1249−1254.
(199) Clouser, D. L.; Jurs, P. C. *Carbohydr. Res.* **1995**, *271*, 65−77.
(200) Mitchell, B. E.; Jurs, P. C. *J. Chem. Inf. Comput. Sci.* **1996**, *36*, 58−64.
(201) de Dios, A. C. *Prog. Nucl. Magn. Reson. Spectrosc.* **1996**, *29*, 229−278.
(202) Havlin, R. H.; Le, H.; Laws, D. D.; de Dios, A. C.; Oldfield, E. *J. Am. Chem. Soc.* **1997**, *119*, 11951−11958.
(203) Helgaker, T.; Jaszunski, M.; Ruud, K. *Chem. Rev.* **1999**, *99*, 293−352.
(204) Tagashira, M.; Yamazaki, S.; Yamanaka, S. *Comput. Mater. Sci.* **1999**, *14*, 248−253.
(205) Wilson, P. J.; Howlin, B. J.; Webb, G. A. *J. Mol. Struct.* **1996**, *385*, 185−193.
(206) St. Hilaire, P. M.; Meldal, M. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 1162−1179.
(207) Schweizer, F.; Hindsgaul, O. *Curr. Opin. Chem. Biol.* **1999**, *3*, 291−298.
(208) Lam, K. S.; Lebl, M.; Krchnak, V. *Chem. Rev.* **1997**, *97*, 411−448.
(209) Shuker, S. B.; Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Science* **1996**, *274*, 1531−1534.
(210) Lin, M.; Shapiro, M. J. *J. Org. Chem.* **1996**, *61*, 7617−7619.
(211) Lin, M.; Shapiro, M. J.; Wareing, J. R. *J. Org. Chem.* **1997**, *62*, 8930−8931.
(212) Chen, A.; Shapiro, M. J. *J. Am. Chem. Soc.* **1998**, *120*, 10258−10259.
(213) Chen, A.; Shapiro, M. J. *J. Am. Chem. Soc.* **2000**, *122*, 414−415.
(214) Meyer, B.; Weimar, T.; Peters, T. *Eur. J. Biochem.* **1997**, *246*, 705−709.
(215) Ni, F. *Prog. Nucl. Magn. Reson. Spectrosc.* **1994**, *26*, 517−606.
(216) Ni, F.; Scheraga, H. A. *Acc. Chem. Res.* **1994**, *27*, 257−264.
(217) Henrichsen, D.; Ernst, B.; Magnani, J. L.; Wang, W.-T.; Meyer, B.; Peters, T. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 98−102.
(218) Herfurth, L.; Weimar, T.; Peters, T. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 2097−2099.
(219) Mayer, M.; Meyer, B. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 1784−1788.
(220) Klein, J.; Meinecke, R.; Mayer, M.; Meyer, B. *J. Am. Chem. Soc.* **1999**, *121*, 5336−5337.
(221) Neiss, T. G.; Cheng, H. N.; Daas, P. J. H.; Schols, H. A. *Am. Chem. Soc. Polym. Prepr.* **1998**, *39*, 688−689.
(222) Abeygunawardana, C.; Williams, T. C.; Summer, J. S.; Hennessey, J. P., Jr. *Anal. Biochem.* **2000**, *279*, 226−240.
(223) Liu, M.; Nicholson, J. K.; Lindon, J. C. *Anal. Chem.* **1996**, *68*, 3370−3376.
(224) Nicholson, J. K.; Foxall, P. J. D.; Spraul, M.; Farrant, R. D.; Lindon, J. C. *Anal. Chem.* **1995**, *67*, 793−811.
(225) Barjat, H.; Morris, G. A.; Smart, S.; Swanson, A. G.; Williams, S. C. R. *J. Magn. Reson, Ser. B* **1995**, *108*, 170−172.
(226) Bäcker, A. E.; Thorbert, S.; Rakotonirainy, O.; Hallberg, E. C.; Olling, A.; Gustavsson, M.; Samuelsson, B. E.; Soussi, B. *Glycoconjugate J.* **1999**, *16*, 45−58.
(227) Lindon, J. C.; Nicholson, J. K. *Trends Anal. Chem.* **1997**, *16*, 190−200.
(228) Wolfender, J.-L.; Rodriguez, S.; Hostettmann, K. *J. Chromatogr. A* **1998**, *794*, 299−316.
(229) Keifer, P. A. *Curr. Opin. Biotechnol.* **1999**, *10*, 34−41.
(230) Malet, C.; Jimenez-Barbero, J.; Bernabe, M.; Brosa, C.; Planas, A. *Biochem. J.* **1993**, *296*, 753−758.
(231) Todeschini, A. R.; Mendonca-Previato, L.; Previato, J. O.; Varki, A.; van Halbeek, H. *Glycobiology* **2000**, *10*, 213−221.

(232) Petersen, B. O.; Krah, M.; Duus, J. Ø.; Thomsen, K. K. *Eur. J. Biochem.* **2000**, *267*, 361−369.
(233) Bock, K. *Pure Appl. Chem.* **1987**, *59*, 1447−1456.
(234) du Penhoat, C. H.; Gey, C.; Pellerin, P.; Perez, S. *J. Biomol. NMR* **1999**, *14*, 253−271.
(235) Vidal, S.; Doco, T.; Williams, P.; Pellerin, P.; York, W. S.; O'Neil, M. A.; Glushka, J.; Darvill, A. G.; Albersheim, P. *Carbohydr. Res.* **2000**, *326*, 277−294.
(236) Olsthoorn, M. M. A.; Petersen, B. O.; Duus, J.; Haverkamp, J.; Thomas-Oates, J. E.; Bock, K.; Holst, O. *Eur. J. Biochem.* **2000**, *267*, 2014−2027.
(237) Holst, O. *Endotoxin in Health and Disease*; Marcel Dekker Inc.: New York, 1999; Chapter 8, pp 115−154.
(238) Flugge, L. A.; Blank, J. T.; Petillo, P. A. *J. Am. Chem. Soc.* **1999**, *121*, 7228−7238.
(239) Jansson, P.-E. *Endotoxin in Health and disease*; Marcel Dekker: New York, 1999; Chapter 9, pp 155−178.
(240) Vinogradov, E.; Bock, K. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 671−674.
(241) Li, X.-C.; Yang, C.-R.; Liu, Y.-Q.; Kasai, R.; Ohtani, K.; Yamasaki, K.; Miyahara, K.; Shingu, K. *Phytochemistry* **1995**, *39*, 1175−1179.
(242) Vinogradov, E.; Bock, K. *Carbohydr. Res.* **1999**, *319*, 92−101.
(243) Vinogradov, E.; Bock, K. *Carbohydr. Res.* **1999**, *320*, 239−243.
(244) Molinaro, A.; de Castro, C.; Petersen, B. O.; Duus, J. Ø.; Parrilli, M.; Holst, O. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 156−160.
(245) Tezuka, Y. *Carbohydr. Res.* **1998**, *305*, 155−161.
(246) Rundlöf, T.; Weintraub, A.; Widmalm, G. *Carbohydr. Res.* **1996**, *291*, 127−139.
(247) Sadovskaya, I.; Brisson, J.-R.; Altman, E.; Mutharia, L. M. *Carbohydr. Res.* **1996**, *283*, 111−127.
(248) Vinogradov, E. V.; Pantophlet, R.; Haseley, S. R.; Brade, L.; Holst, O.; Brade, H. *Eur. J. Biochem.* **1997**, *243*, 167−173.
(249) Leslie, M. R.; Parolis, H.; Parolis, L. A. S.; Petersen, B. O. *Carbohydr. Res.* **1998**, *309*, 95−101.
(250) Uhrin, D.; Chandan, V.; Altman, E. *Can. J. Chem.* **1995**, *73*, 1600−1604.
(251) Haseley, S. R.; Holst, O.; Brade, H. *Eur. J. Biochem.* **1997**, *244*, 761−766.
(252) Kocharova, N. A.; Shcherbakova, O. V.; Shashkov, A. S.; Knirel, Y. A.; Kochetkov, N. K.; Kholodkova, E. V.; Stanislavsky, E. S. *Biochemistry (Moscow)* **1997**, *62*, 501−508.
(253) Iacomini, M.; Casu, B.; Guerrini, M.; Naggi, A.; Pirola, A.; Torri, G. *Anal. Biochem.* **1999**, *274*, 50−58.
(254) Miller, I. J.; Blunt, J. W. *Botanica Marina* **2000**, *43*, 239−250.
(255) Knirel, Y. A.; Grosskurth, H.; Helbig, J. H.; Zähringer, U. *Carbohydr. Res.* **1995**, *279*, 215−226.
(256) Parolis, L. A. S.; Duus, J. Ø.; Parolis, H.; Meldal, M.; Bock, K. *Carbohydr. Res.* **1996**, *293*, 101−117.
(257) Helander, I. M.; Kilpeläinen, I.; Vaara, M. *FEBS Lett.* **1997**, *409*, 457−460.
(258) Parolis, L. A. S.; Parolis, H.; Kenne, L.; Meldal, M.; Bock, K. *Carbohydr. Res.* **1998**, *309*, 77−87.
(259) Gunawardena, S.; Fiore, C. R.; Johnson, J. A.; Bush, C. A. *Biochemistry* **1999**, *38*, 12062−12071.
(260) Jachymek, W.; Czaja, J.; Niedziela, T.; Lugowski, C.; Kenne, L. *Eur. J. Biochem.* **1999**, *266*, 53−61.
(261) de Waard, P.; Vliegenthart, J. F. G. *J. Magn. Reson.* **1989**, *81*, 173−177.
(262) De Bruyn, A.; Maras, M.; Schraml, J.; Herdewijn, P.; Contreras, R. *FEBS Lett.* **1997**, *405*, 111−113.
(263) Vinogradov, E.; Petersen, B. O.; Duus, J. Ø. *Carbohydr. Res.* **2000**, *325*, 216−221.
(264) Gotfredsen, C. H.; Meissner, A.; Duus, J. Ø.; Sørensen, O. W. *Magn. Reson. Chem.* **2000**, *38*, 692−695.
(265) Schraml, J.; De Bruyn, A.; Contreras, R.; Herdewijn, P. *J. Carbohydr. Chem.* **1997**, *16*, 165−170.
(266) Summers, M. F.; Marzilli, L. G.; Bax, A. *J. Am. Chem. Soc.* **1986**, *108*, 4285−4294.
(267) Jones, D. N. M.; Sanders, J. K. M. *J. Chem. Soc., Chem. Commun.* **1989**, 167−169.
(268) Jones, D. N. M.; Sanders, J. K. M. *J. Am. Chem. Soc.* **1989**, *111*, 5132−5137.
(269) Knijn, A.; Casieri, C.; Carpinelli, G.; Testa, C.; Podo, F.; De Luca, F. *NMR Biomed.* **2000**, *13*, 124−128.
(270) Fiaux, J.; Andersson, C. I. J.; Holmberg, N.; Bülow, L.; Kallio, P. T.; Szyperski, T.; Bailey, J. E.; Wüthrich, K. *J. Am. Chem. Soc.* **1999**, *121*, 1407−1408.
(271) Heydorn, A.; Petersen, B. O.; Duus, J. Ø.; Bergmann, S.; Suhr-Jessen, T.; Nielsen, J. *J. Biol. Chem.* **2000**, *275*, 6201−6206.
(272) Schmidt, K.; Nørregaard, L. C.; Pedersen, B.; Meissner, A.; Duus, J. Ø.; Nielsen, J. Ø.; Villadsen, J. *Metabol. Eng.* **1999**, *1*, 166−179.
(273) Tavernier, P.; Besson, I.; Portais, J.-C.; Courtois, J.; Courtois, B.; Barbotin, J.-N. *Biotechnol. Bioeng.* **1998**, *58*, 250−253.
(274) Bank, S.; Yan, B.; Miller, T. L. *Solid State Nucl. Magn. Reson.* **1996**, *7*, 253−261.
(275) Tavernier, P.; Portais, J.-C.; Besson, I.; Courtois, J.; Courtois, B.; Barbotin, J.-N. *J. Chim. Phys.* **1998**, *95*, 256−259.
(276) Kjellberg, A.; Weintraub, A.; Widmalm, G. *Biochemistry* **1999**, *38*, 12205−12211.
(277) Kai, A.; Karasawa, H.; Kikawa, M.; Hatanaka, K.; Matsuzaki, K.; Mimura, T.; Kaneko, Y. *Carbohydr. Polym.* **1998**, *35*, 271−278.
(278) Chung, J.; Tolman, J. R.; Howard, K. P.; Prestegard, J. H. *J. Magn. Reson, Ser. B* **1993**, *102*, 137−147.
(279) Gosser, Y. Q.; Howard, K. P.; Prestegard, J. H. *J. Magn. Reson, Ser. B* **1993**, *101*, 126−133.
(280) Harris, R.; Rutherford, T. J.; Milton, M. J.; Homans, S. W. *J. Biomol. NMR* **1997**, *9*, 47−54.
(281) Gilhespy-Muskett, A. M.; Partridge, J.; Jefferis, R.; Homans, S. W. *Glycobiology* **1994**, *4*, 485−489.
(282) Yu, L.; Goldman, R.; Sullivan, P.; Walker, G. F.; Fesik, S. W. *J. Biomol. NMR* **1993**, *3*, 429−441.
(283) Sheng, S.; Cherniak, R. *Carbohydr. Res.* **1997**, *301*, 33−40.
(284) Linnerborg, M.; Weintraub, A.; Widmalm, G. *Eur. J. Biochem.* **1999**, *266*, 246−251.
(285) Kjellberg, A.; Nishida, T.; Weintraub, A.; Widmalm, G. *Magn. Reson. Chem.* **1998**, *35*, 128−131.
(286) Bax, A.; Clore, G. M.; Driscoll, P. C.; Gronenborn, A. M.; Ikura, M.; Kay, L. E. *J. Magn. Reson.* **1990**, *87*, 620−627.
(287) Xu, Q.; Bush, C. A. *Carbohydr. Res.* **1998**, *306*, 335−339.
(288) Xu, Q.; Bush, C. A. *Biochemistry* **1996**, *35*, 14512−14520.
(289) Martin-Pastor, M.; Bush, C. A. *Biochemistry* **1999**, *38*, 8045−8055.
(290) Lacey, M. E.; Subramanian, R.; Olson, D. L.; Webb, A. G.; Sweedler, J. V. *Chem. Rev.* **1999**, *99*, 3133−3152.
(291) Christensen, M. K.; Meldal, M.; Bock, K.; Cordes, H.; Mouritsen, S.; Elsner, H. *J. Chem. Soc., Perkin Trans. 1* **1994**, 1299−1310.
(292) Meldal, M.; Christiansen-Brams, I.; Christensen, M. K.; Mouritsen, S.; Bock, K. *Complex Carbohydrates in Drug Research; Structural and Functional Aspects*; Munksgaard: Copenhagen, 1994; pp 153−164.
(293) Wu, S.-H.; Shimazaki, M.; Lin, C.-C.; Qiao, L.; Moree, W. J.; Weitz-Schmidt, G.; Wong, C.-H. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 88−90.
(294) Shoolery, J. N. *Top. Carbon-13 NMR Spectrosc.* **1979**, *3*, 28−38.
(295) Odelblad, E. *Acta Obstet. Gynecol. Scand.* **1966**, *65 (supl. 2)*, 7−188.
(296) Crouch, R. C.; Martin, G. E. *Magn. Reson. Chem.* **1992**, *30*, S66−S70.
(297) Reynolds, W. F.; Yu, M.; Enriquez, R. G. *Magn. Reson. Chem.* **1997**, *35*, 614−618.
(298) Martin, G. E.; Guido, J. E.; Robins, R. H.; Sharaf, M. H. M.; Schiff, P. L., Jr.; Tackie, A. N. *J. Nat. Prod.* **1998**, *61*, 555−559.
(299) Subramanian, R.; Lam, M. M.; Webb, A. G. *J. Magn. Reson.* **1998**, *133*, 227−231.
(300) Martin, G. E.; Hadden, C. E. *Magn. Reson. Chem.* **1999**, *37*, 721−729.
(301) Subramanian, R.; Webb, A. G. *Anal. Chem.* **1998**, *70*, 2454−2458.
(302) Li, Y.; Wolters, A. M.; Malawey, P. V.; Sweedler, J. V.; Webb, A. G. *Anal. Chem.* **1999**, *71*, 4815−4820.
(303) Hoult, D. I.; Richards, R. E. *J. Magn. Reson.* **1976**, *24*, 71−85.
(304) Shapiro, M. J.; Wareing, J. R. *Curr. Opin. Chem. Biol.* **1998**, *2*, 372−375.
(305) Keifer, P. A. *Drugs Future* **1998**, *23*, 301−317.
(306) Keifer, P. A. *Drug Discovery Today* **1997**, *2*, 468−478.
(307) Subramanian, R.; Kelley, W. P.; Floyd, P. D.; Tan, Z. J.; Webb, A. G.; Sweedler, J. V. *Anal. Chem.* **1999**, *71*, 5335−5339.
(308) Olson, D. L.; Lacey, M. E.; Webb, A. G.; Sweedler, J. V. *Anal. Chem.* **1999**, *71*, 3070−3076.
(309) Barbara, T. M. *J. Magn. Reson., Ser. A* **1994**, *109*, 265−269.
(310) Fitch, W. L.; Detre, G.; Holmes, C. P.; Shoolery, J. N.; Keifer, P. A. *J. Org. Chem.* **1994**, *59*, 7955−7956.
(311) Lippens, G.; Bourdonneau, M.; Dhalluin, C.; Warrass, R.; Richert, T.; Seetharaman, C.; Boutillon, C.; Piotto, M. *Curr. Org. Chem.* **1999**, *3*, 147−169.
(312) Brus, J. *Solid State Nucl. Magn. Reson.* **2000**, *16*, 151−160.
(313) Kupce, E.; Keifer, P. A.; Delepierre, M. *J. Magn. Reson.* **2000**, in press.
(314) Maas, W. E.; Laukien, F. H.; Cory, D. G. *J. Am. Chem. Soc.* **1996**, *118*, 13085−13086.
(315) Keifer, P. A.; Baltusis, L.; Rice, D. M.; Tymiak, A. A.; Shoolery, J. N. *J. Magn. Reson., Ser. A* **1996**, *119*, 65−75.
(316) Broberg, A.; Kenne, L.; Pedersen, M. *Planta* **1998**, *206*, 300−307.
(317) Broberg, A.; Kenne, L. *Anal. Biochem.* **2000**, *284*, 367−374.
(318) Sayer, B. G.; Preston, C. M. *Seed Sci. Technol.* **1996**, *24*, 321−329.
(319) Krainer, E.; Stark, R. E.; Naider, F.; Alagramam, K.; Becker, J. M. *Biopolymers* **1994**, *34*, 1627−1635.
(320) Cheng, L. L.; Chang, I.-W.; Louis, D. N.; Gonzalez, R. G. *Cancer Res.* **1998**, *58*, 1825−1832.
(321) Cheng, L. L.; Ma, M. J.; Becerra, L.; Ptak, T.; Tracey, I.; Lackner, A.; González, R. G. *Proc. Nat. Acad. Sci. U.S.A.* **1997**, *94*, 6408−6413.

(322) Chin, J.; Fell, B.; Shapiro, M. J.; Tomesch, J.; Wareing, J. R.; Bray, A. M. *J. Org. Chem.* **1997**, *62*, 538−539.
(323) Dhalluin, C.; Boutillon, C.; Tartar, A.; Lippens, G. *J. Am. Chem. Soc.* **1997**, *119*, 10494−10500.
(324) Seeberger, P. H.; Beebe, X.; Sukenick, G. D.; Pochapsky, S.; Danishefsky, S. J. *Angew. Chem., Int. Ed. Engl.* **1997**, *36*, 491−493.
(325) Styles, P.; Soffe, N. F.; Scott, C. A. *J. Magn. Reson.* **1989**, *84*, 376−378.
(326) Serber, Z.; Richter, C.; Moskau, D.; Böhlen, J.-M.; Gerfin, T.; Marek, D.; Häberli, M.; Baselgia, L.; Laukien, F.; Stern, A. S.; Hoch, J. C.; Dötsch, V. *J. Am. Chem. Soc.* **2000**, *122*, 3554−3555.
(327) Styles, P.; Soffe, N. F.; Scott, C. A.; Cragg, D. A.; Row: F.; White, D. J.; White, P. C. J. *J. Magn. Reson.* **1984**, *60*, 397−404.
(328) Anderson, W. A.; Brey, W. W.; Brooke, A. L.; Cole, B.; Delin, K. A.; Fuks, L. F.; Hill, H. D. W.; Johanson, M. E.; Kotsubo, V. Y.; Nast, R.; Withers, R. S.; Wong, W. H. *Bull. Magn. Reson.* **1995**, *17*, 98−102.
(329) Hill, H. D. W. *IEEE Trans. Appl. Superconductivity* **1997**, *7*, 3750−3755.
(330) Flynn, P. F.; Mattiello, D. L.; Hill, H. D. W.; Wand, A. J. *J. Am. Chem. Soc.* **2000**, *122*, 4823−4824.
(331) Hajduk, P. J.; Gerfin, T.; Boehlen, J.-M.; Häberli, M.; Marek, D.; Fesik, S. W. *J. Med. Chem.* **1999**, *42*, 2315−2317.
(332) Hajduk, P. J.; Meadows, R. P.; Fesik, S. W. *Q. Rev. Biophys.* **1999**, *32*, 211−240.
(333) Logan, T. M.; Murali, N.; Wang, G.; Jolivet, C. *Magn. Reson. Chem.* **1999**, *37*, 762−765.
(334) Bringmann, G.; Wohlfarth, M.; Rischer, H.; Grüne, M.; Schlauer, J. *Angew. Chem., Int. Ed. Engl.* **2000**, *39*, 1464−1466.
(335) Rund, S.; Lindner, B.; Brade, H.; Holst, O. *J. Biol. Chem.* **1999**, *274*, 16819−16824.
(336) Manzi, A.; Salimath, P. V.; Spiro, R. C.; Keifer, P. A.; Freeze, H. H. *J. Biol. Chem.* **1995**, *270*, 9154−9163.
(337) Manzi, A. E.; Keifer, P. A. *Techniques in Glycobiology*; Marcel Dekker Inc.: New York, 1997; Chapter 1, pp 1−16.
(338) Kitagawa, H.; Tanaka, Y.; Tsuchida, K.; Goto, F.; Ogawa, T.; Lidholt, K.; Lindahl, U.; Sugahara, K. *J. Biol. Chem.* **1995**, *270*, 22190−22195.
(339) Gilbert, M.; Brisson, J.-R.; Karwaski, M.-F.; Michniewicz, J.; Cunningham, A.-M.; Wu, Y.; Young, N. M.; Wakarchuk, W. W. *J. Biol. Chem.* **2000**, *275*, 3896−3906.
(340) Broberg, A.; Thomsen, K. K.; Duus, J. Ø. *Carbohydr. Res.* **2000**, *328*, 375−382.
(341) Gotfredsen, C. H.; Grøtli, M.; Willert, M.; Meldal, M.; Duus, J. Ø. *J. Chem. Soc., Perkin Trans. 1* **2000**, 1167−1171.
(342) Meiboom, S.; Gill, D. *Rev. Sci. Instrum.* **1958**, *29*, 688.
(343) Jachymek, W.; Niedziela, T.; Peterson, C.; Lugowski, C.; Czaja, J.; Kenne, L. *Biochemistry* **1999**, *38*, 11788−11795.
(344) Czaja, J.; Jachymek, W.; Niedziela, T.; Lugowski, C.; Aldova, E.; Kenne, L. *Eur. J. Biochem.* **2000**, *267*, 1672−1679.
(345) Haase, W.-C.; Seeberger, P. H. *Curr. Org. Chem.* **2000**, *4*, 481−511.
(346) Kanemitsu, T.; Kanie, O.; Wong, C.-H. *Angew. Chem., Int. Ed. Engl.* **1998**, *37*, 3415−3418.
(347) Sears, P.; Wong, C.-H. *Angew. Chem., Int. Ed. Engl.* **1999**, *38*, 2301−2324.
(348) Halkes, K. M.; St. Hilaire, P. M.; Jansson, A. M.; Gotfredsen, C. H.; Meldal, M. *J. Chem. Soc., Perkin Trans. 1* **2000**, 2127−2133.
(349) Renil, M.; Meldal, M. *Tetrahedron Lett.* **1996**, *37*, 6185−6188.
(350) Grøtli, M.; Gotfredsen, C. H.; Rademann, J.; Buchardt, J.; Clark, A. J.; Duus, J. Ø.; Meldal, M. *J. Comb. Chem.* **2000**, *2*, 108−119.
(351) Keifer, P. A. *J. Org. Chem.* **1996**, *61*, 1558−1559.
(352) Markiewicz, W. D.; Dixon, I. R.; Eyssa, Y. M.; Swenson, C. A.; Schneider-Muntau, H. J. *High Magnetic Fields: Applications, Generation, Materials*; World Scientificfic: NJ, 1997; pp 287−295.
(353) Markiewicz, W. D. *Solid State Nucl. Magn. Reson.* **1997**, *9*, 73−76.
(354) Brooks, J. S.; Crow, J. E.; Moulton, W. G. *J. Phys. Chem. Solids* **1998**, *59*, 569−590.
(355) Keifer, P. A.; Smallcombe, S. H.; Williams, E. H.; Salomon, K. E.; Mendez, G.; Belletire, J. L.; Moore, C. D. *J. Comb. Chem.* **2000**, *2*, 151−171.

CR990302N