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Analysis of Carbohydrate Mixtures by Diffusion Difference NMR Spectroscopy

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Summary. Diffusion difference NMR spectroscopy can be used as a fast and powerful tool to separate carbohydrates in a mixture by their translational diffusion properties. This method is of general interest for analyzing natural and synthetic mixtures and for monitoring and optimizing synthetic reactions. The proposed subtraction procedure applied to two varying diffusion encoded spectra can be combined with homo- and heteronuclear as well as with one- and multi-dimensional NMR experiments.

Keywords. NMR spectroscopy; Carbohydrates; Diffusion difference; BPP-LED.

Introduction

The structural investigation of carbohydrates by nuclear magnetic resonance (NMR) spectroscopy is still a very challenging field even at the highest available magnetic field strengths (for a recent review, see Ref. [1]). Additionally, nowadays not only purified samples containing one isolated compound are analyzed; there is an increasing demand on the determination of the composition of complex mixtures by spectroscopic techniques, its main reason being the effort to increase efficiency and throughput in drug discovery processes. Combinatorial chemistry techniques have been developed to create complex libraries of molecules with the necessity of testing and identifying the individual compounds as mixtures; structure-activity relationships (SAR) by NMR is an example for a strategy developed for screening mixtures for biological activity [2]. In a similar way, natural products, which are applied as a rich source of useful compounds, involve the identification of interesting molecules in a biological matrix. Analytical tools for controlling the success of a synthetic process at a very early stage are also desirable in 'classical' organic chemistry. Thus, the main issue is avoiding expensive and time consuming physical separation of the mixtures prior to analysis.

In contrast to the majority of spectroscopic techniques, NMR spectroscopy in combination with pulsed field gradients (PFG) can lead to a non-invasive structural investigation of unknown compounds in complex mixtures. These experiments rely on the attenuation behaviour of individual resonances under the influence of linear field gradients due to translational diffusion [3]. Since diffusion, in addition to

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effects exerted by bulk parameters, is a property of a molecule as a whole, individual mixture components can be resolved based on the variance of their diffusion rates using diffusion ordered (DOSY; for a recent review, see Ref. [4]) or diffusion decoded (DECODES, [5–8]) NMR spectroscopy. After calibration of the PFG amplitudes and fitting of the resulting lineshapes, the individual diffusion coefficients can be derived for different sized molecules or, because of differences in hydrodynamic radii, even for molecules of the same molecular weight [9].

A few applications of DOSY experiments have already been reported in the field of carbohydrate NMR spectroscopy. These include complexation studies of monosaccharides with lanthanide cations [10], the investigation of silylated mono- and disaccharides [11], and measurement of the diffusion constants of nucleic acids for assessing whether a monomer or a duplex is present [12]. A qualitative approach has been published for identifying low molecular weight impurities in synthetic pentasaccharide samples [13]. In this paper we present diffusion difference NMR spectroscopy as our contribution to carbohydrate mixture analysis resulting from the demand for a fast, non-invasive, and easily workable spectroscopic tool of very broad applicability.

Results and Discussion

The concept of diffusion difference NMR spectroscopy

In fact, for the practical use in the routine analysis of carbohydrate mixtures the above mentioned DOSY or DECODES related experiments exhibit several drawbacks. First of all, they are rather time consuming as a series of one- or two-dimensional NMR spectra have to be recorded to resolve the molecules by their diffusion properties. New approaches recently published are very promising, accomplishing *e.g.* diffusion measurements within a single scan (DIFFTRAIN) [14] or a one-dimensional DOSY by using a nonuniform magnetic field gradient to encode the diffusion information [15]. Nevertheless, the analysis of the resulting spectra, which consists of applying appropriate line fitting algorithms to individual peaks belonging to different compounds for decoding the diffusion constants, still remains. Secondly, the gradient amplitude has to be calibrated very accurately; otherwise, only relative diffusion coefficients can be measured. Convection currents resulting from small temperature gradients within the sample tube can cause additional signal decay [16, 17], thus hampering the quantitative interpretation of the spectra.

As for the NMR analysis of mixtures the accurate determination of the diffusion coefficients of the individual components is not of main interest, we propose a general and faster qualitative diffusion difference approach by combining an old concept [18] with modern stimulated echo experiments. In this case, instead of a series only two NMR spectra will be acquired, using as a diffusion encoding element the bipolar-gradient pulses longitudinal eddy-current delayed sequence (BPP-LED) [19]. The first spectrum will be recorded with a very weak gradient amplitude, the second one with a somewhat stronger, regardless of its absolute value. The resulting spectra, showing differently attenuated peaks due to the distinct diffusion properties of individual compounds, are then subtracted. Depending on the proper scaling of their relative intensities, the difference trace gives the low or the

high molecular weight compounds, respectively. As main advantages over DOSY type experiments, diffusion difference NMR spectroscopy is fast, no calibration of the gradient amplitude is necessary, and the postprocessing consists only of a rather simple subtraction of two spectra. Additionally, the receiver gain can be optimized for every single experiment depending on the chosen gradient amplitude, thus giving better signal-to-noise ratios. This fact is of major importance, since the overall signal intensity of the whole spectrum will be attenuated in any case by increasing the gradient amplitude. The following selected examples for the analysis of natural or synthetically derived carbohydrate mixtures using modern one- (1D) as well as two-dimensional (2D) NMR techniques will demonstrate the general and fast applicability of diffusion difference NMR spectroscopy.

Analysis of a mixture of homoglycans

A mixture of the α-(1$\rightarrow$4)-linked glucose oligomers maltose and maltotriose of about equal amounts on the milligram scale should illustrate the concept of the diffusion difference NMR procedure. The resonance signals for the disaccharide in a stimulated echo ^{1}H NMR spectrum should be significantly reduced compared to the signals of the trisaccharide. As the structural similarity of these two homoglycans, which differ only in chain length, leads to almost identical NMR spectra, high demands have to be made on the experimental conditions in order to resolve the components by a subtraction method. For spectrometer stability reasons, the bipolar-gradient pulses LED sequence [19] was chosen for diffusion encoding. Figure 1 shows expansions of the resulting spectra recorded with 32 scans using 5% and 80% gradient amplitudes, respectively. Scaling the signal intensities for the higher molecular weight compound to an equal level and subtracting the spectrum derived with higher gradient strength from that obtained with the lower one results in a positive difference trace for all faster moving compounds, in this case maltose and the solvent. Figure 1 shows also a comparison between the diffusion difference NMR spectrum (trace c) and a normal ^{1}H NMR spectrum of pure maltose (trace d). Signals resonating very closely together like the non-reducing anomeric protons and especially H-4 (assignments taken from Ref. [20]) from the non-reducing end glucopyranoses with a chemical shift difference between maltose and maltotriose of only 1.8 Hz can be resolved in the mixture without any distortions.

Peptidoglycan analysis

An illustrative example for the application of this fast one-dimensional NMR carbohydrate mixture analysis procedure derives from the characterization of the linkage region between a bacterial cell wall polysaccharide and the surface-layer (S-layer) protein of *Thermoanaerobacterium thermosaccharolyticum* D120-70 [21]. After proteolytic degradation of the S-layer glycoprotein, tyrosine was found as the linking amino acid; however, the signals of the repeating units consisting of six residues obscured the information of the linkage sugar to the protein. In order to reduce the polysaccharide background, a mild acidic degradation of the glycopeptide was performed, yielding – after a crude separation – an NMR sample suitable for a fast diffusion edited experiment. After scaling the intensities of the

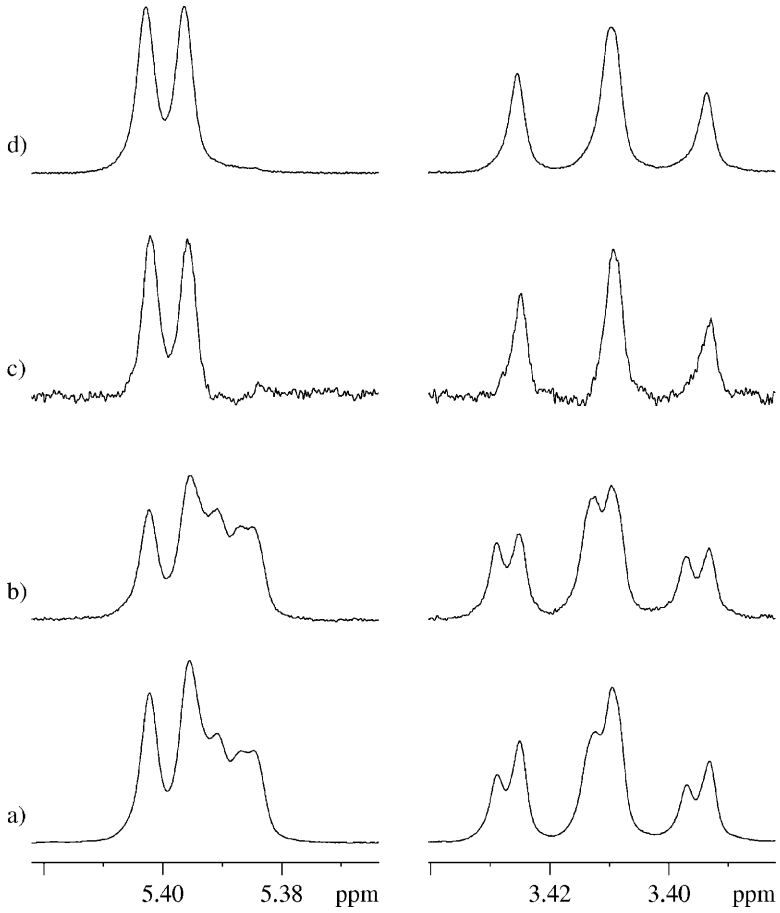

Fig. 1. BPP-LED spectra of a maltose/maltotriose mixture showing the non-reducing anomeric protons in the left and H-4 from the non-reducing end glucopyranose in the right column; a) recorded with 5%, b) with 80% gradient amplitudes, c) diffusion difference spectrum, d) normal ^{1}H NMR spectrum of pure maltose

signals for the central repeats to equal height, the diffusion difference experiment results in a clean and straightforwardly assignable ^{1}H NMR spectrum of the O-glycosidic linkage region β-*D*-glucopyranose-O→tyrosine (Fig. 2).

The BPP-LED pulse sequence together with the subtraction procedure is also suitable for addressing another problem connected to natural polysaccharide samples. The chain length of the aforementioned intact peptidoglycan can be determined by integration of the ^{1}H NMR spectrum. By comparing the intensities of the well-separated anomeric signals with those of the aromatic protons of tyrosine it could be deduced that the peptidoglycan consists of seven repeating units. However, the diffusion difference spectrum obtained after scaling the intensities of signals belonging to terminal sugar residues and to the amino acid to an equal level still shows the resonance lines of the central repeats (Fig. 3). Thus, the chain length of the polysaccharide is only on the average seven repeating units, and the BPP-LED NMR spectra supply evidence for a natural microheterogeneity of the isolated peptidoglycan. A matrix-assisted laser desorption ionization time-of-flight

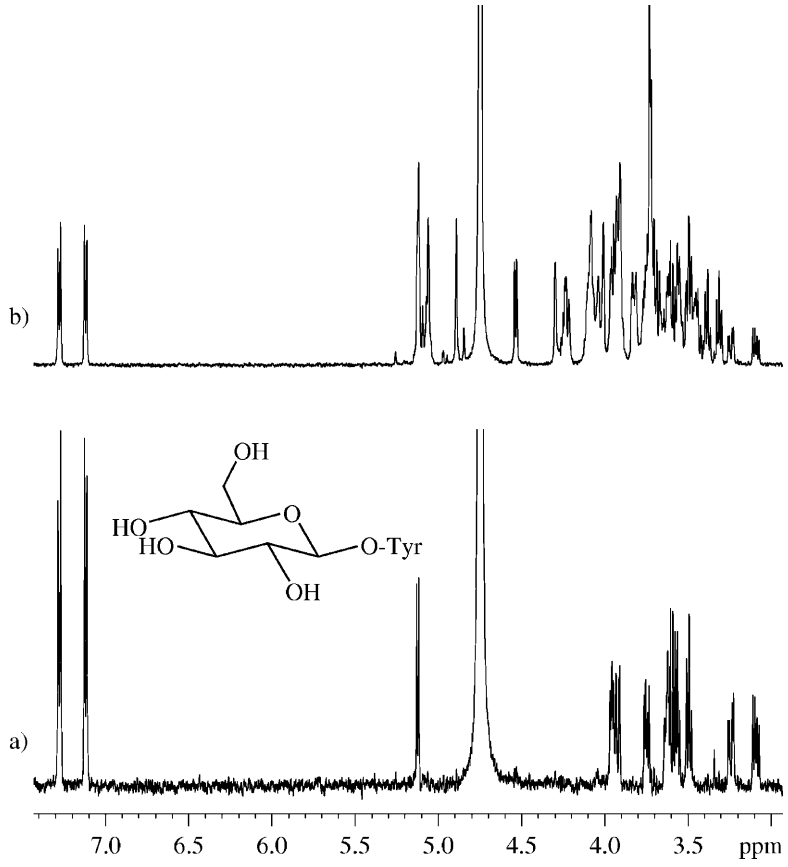

Fig. 2. Partly degraded bacterial cell wall peptidoglycan: a) diffusion difference NMR spectrum (5% and 50% gradient amplitudes), b) ^{1}H NMR spectrum

(MALDI-TOF) mass spectrum confirmed this chain length variability, showing five to nine hexasaccharide repeats linked to the tyrosine [21].

Derivatization of adenosine monophosphate (AMP)

Diffusion difference NMR spectroscopy proved to be a powerful tool for the quick monitoring of the success of the chemical synthesis of 2′(3′)-O-(N-acylaminoacyl)-nucleotide derivatives of adenosine-5′-monophosphate as well as of the corresponding *AMP*-3′-thio analogues. These modified mononucleotides were used to investigate the peptidyl transferase activity in ribosomes. Simple aminoacylated analogues of the 3′-terminal adenosine of transfer ribonucleic acid (t*RNA*) possess donor activity in the peptidyl transferase reaction, provided that they are used at very high concentrations (about 1 m*M*) [22]. To study the substrate specificity of the peptidyl transferase center as well as to investigate functional groups which might be involved in the chemical step, several differently modified mononucleotide substrates were synthesized. The success of the preparation of aminoacylated *AMP* derivatives and the optimization of the synthetic process can be investigated only by mixture analysis, as even after reversed phase high performance

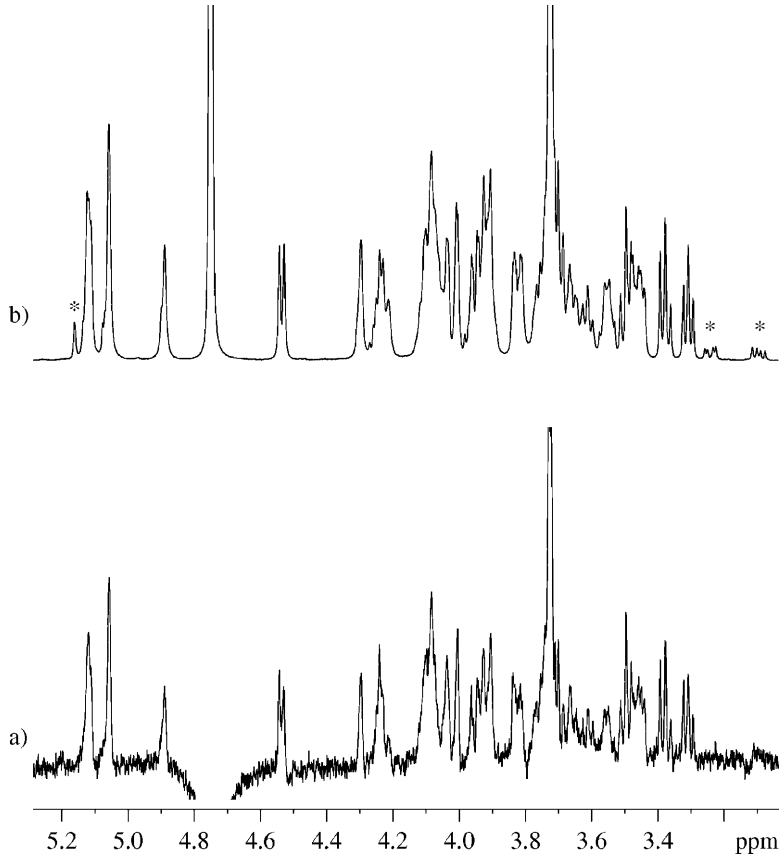

Fig. 3. Intact peptidoglycan: a) diffusion difference NMR spectrum (5% and 55% gradient amplitudes, terminal signal intensities scaled to equal level), b) ^{1}H NMR spectrum (signals from terminal residues marked with $*$)

liquid chromatography (HPLC) purification no fraction contained only one single product. This problem does not arise mainly from comigration on the column but is rather due to the low stability of the ester bond between amino acid and nucleotide. Furthermore, the interconversion between the 2′- and 3′-hydroxyl groups of adenosine is rapid in aqueous buffer solution. An even more complex pattern was detected by the use of a sulfur containing mononucleotide resulting from dimerization products due to disulfide formation.

Nevertheless, many of the possible components in the reaction mixture are of different molecular weight and should be distinguishable by their translational diffusion properties. Therefore, diffusion difference NMR spectroscopy can be used as a fast and easy tool to prove whether *e.g.* the ester bond between *AMP* and the acylamino acid is formed by the chosen reaction conditions or not. Figure 4 gives an example of the mixture obtained after a successful acylation of *AMP* with N-acetylphenylalanin cyanomethylester. In the chemical shift region for the anomeric protons five resonance signals can be seen, indicating five different *AMP* species. In the diffusion difference NMR spectrum (trace b), only a positive trace for the excessive free N-acetylphenylalanin and for the fast moving solvents (HDO and

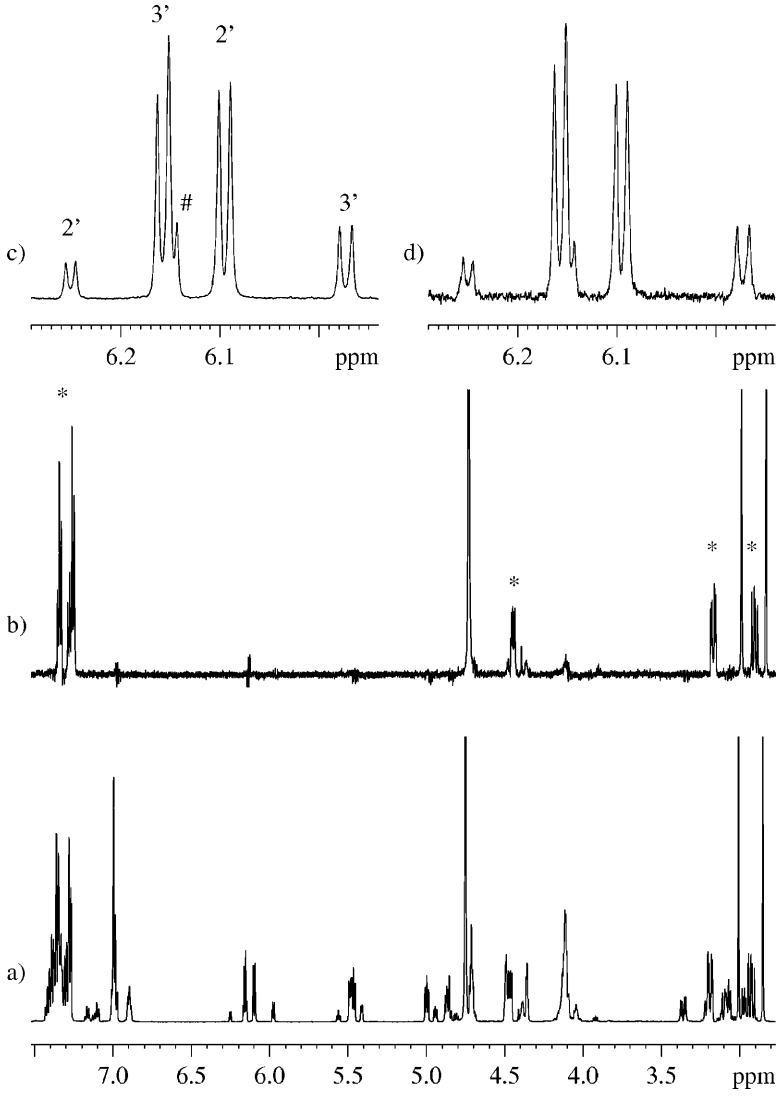

Fig. 4. Reaction mixture from the derivatization of *AMP* with N-acetyl-phenylalanine (Ac-*Phe*); a) ^{1}H NMR spectrum, b) diffusion difference spectrum showing free Ac-*Phe* (signals marked with *), c) BPP-LED spectrum with 5% and d) with 40% gradient amplitudes (2′- and 3′-substituted *AMP* with racemization of Ac-*Phe*; free *AMP* marked with #)

DMF) can be observed after scaling the anomeric signals to equal height. Looking closer to the H-1′ shift region, the weak doublet at 6.15 ppm decays significantly faster by applying stronger gradients than the other four signals (traces c and d). Although this signal as well as the rest of the related ribose protons cannot easily be seen in the difference trace due to their very low intensity and the poor signal-to-noise ratio after the subtraction, this anomeric proton belongs to the faster moving free *AMP*. The other four H-1′ signals with almost identical diffusion behaviour are part of the monoacylated ribose 2′- and 3′-derivatives, including racemization products of the amino acid. Negative signals in the difference trace b indicating higher

molecular weight compounds can be seen only very weakly for the main 3′-aminoacylated adenosine, which is most likely due to different hydrodynamic radii of the 2′- and 3′-isomers. In any case, no disubstituted *AMP* was formed. All assignments were confirmed by additional NMR experiments. However, applying diffusion difference NMR spectroscopy for this carbohydrate mixture analysis was the best choice for a very fast optimization of the synthesis of the desired acylated *AMP* substrates and of their thio-analogues.

Two-dimensional diffusion difference NMR spectroscopy

The combination of diffusion difference NMR spectroscopy with homo- and heteronuclear two-dimensional NMR experiments is shown in the last part. Again the BPP-LED pulse sequence was used as diffusion encoding element, followed by a total correlation experiment (TOCSY) giving the improved DECODES pulse sequence [8] or by a sensitivity enhanced heteronuclear single quantum coherence experiment (HSQC) resulting in the HETDECODES pulse sequence [8]. After the acquisition of two spectra using different gradient amplitudes, 1D traces containing the signals of interest have to be read out of the 2D matrix in order to adjust the vertical scaling for the subtraction process. Figure 5 shows heavily overlapping cross-peaks for the non-reducing anomeric signals of the homoglycan mixture maltose and maltotriose. Diffusion difference spectra derived after scaling the cross-peak

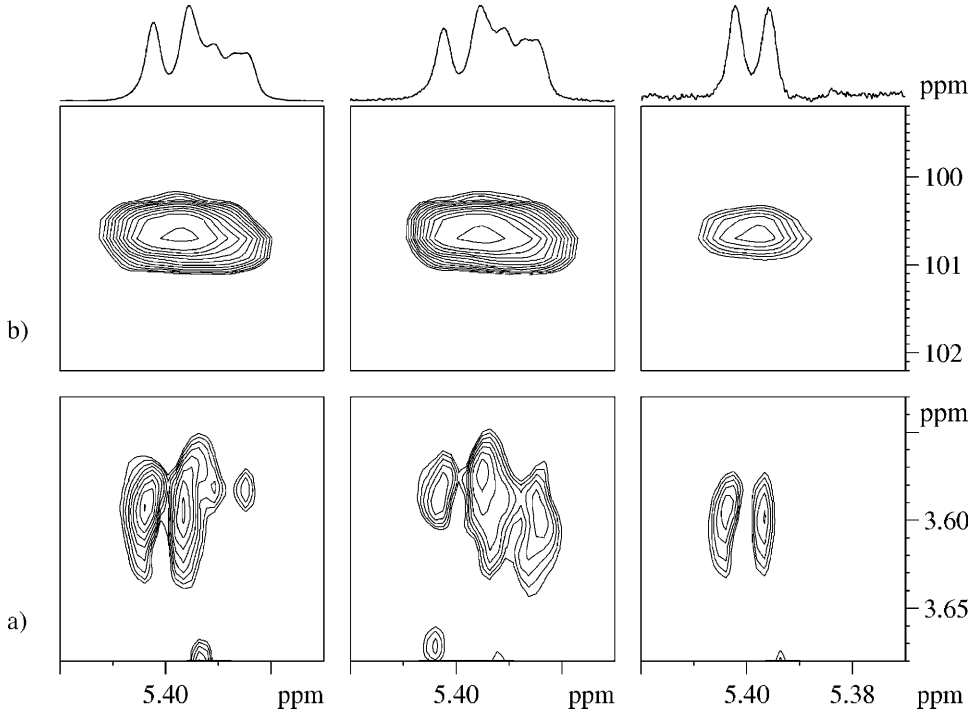

Fig. 5. Expansions from 2D diffusion edited NMR spectra of the non-reducing anomeric signals of a maltose/maltotriose mixture; a) improved DECODES experiment, b) HETDECODES experiments with 3% (left) and 50% (middle) gradient amplitudes and diffusion difference spectra (right)

intensities for the higher molecular weight compound to equal height and subtraction of two DECODES or of two HETDECODES spectra results in clean cross-peaks for the maltose, thus demonstrating the power of this technique even for the mixture analysis of very similar structures with almost identical ^{1}H and ^{13}C chemical shifts.

Conclusions

Diffusion difference NMR spectroscopy was shown to be a powerful tool in carbohydrate mixture analysis. It is suitable for addressing a wide variety of problems, like characterization of known and unknown compounds, identification of microheterogeneity in natural polysaccharide samples, and controlling and optimization of synthetic reactions. It is a very fast non-invasive tool covering 1D as well as 2D homo- and heteronuclear NMR experiments. Moreover, its application is not limited to carbohydrate mixture analysis.

Experimental

All NMR spectra were recorded on a Bruker Avance DRX 600 NMR spectrometer using a 5 mm inverse triple probe (^{1}H, ^{13}C, broad-band) with triple axis gradient coils. The samples were dissolved in 0.6 cm^3 D_2O and were measured at a temperature of 300 K with a resonance frequency of 600.13 MHz for ^{1}H and 150.90 MHz for ^{13}C. The amounts of the samples were on the milligram scale (homoglycan mixture: 3.3 mg maltose, 3.5 mg maltotriose; intact peptidoglycan sample: 4.3 mg, partial degraded peptidoglycan mixture: 1 mg; mixtures of the *AMP* derivatization reactions: approximately 10 mg). The spectra were referenced for ^{1}H to the internal HDO solvent signal ($\delta =$ 4.75 ppm), for ^{13}C to external dioxane ($\delta = 67.40$ ppm).

In all experiments, the diffusion encoding was performed with the BPP-LED sequence [19] with 1 ms sinusoidal bipolar-gradient pulse pairs; the diffusion delay was set to 150 ms. The gradient amplitudes are given directly in the figure captions (Acustar II gradient amplifier, 3×10 A, z-gradient: $g_{max} = 60$ G/cm). The number of scans for the 1D spectra was in the range from 32 to 512 depending on the relative intensities of the signals from the individual mixture components, using more scans if weak signals were of interest. The TOCSY experiment was performed using the improved DECODES pulse sequence [8] with a spin lock time of 100 ms collecting 2 k data points in f2 and 256 increments with 8 scans each in f1. For the HSQC experiment, the HETDECODES pulse sequence [8] was used collecting 2 k data points in f2 and 128 increments with 32 scans each in f1.

All processing was done off-line on a Silicon Graphics O2 workstation using the Bruker software Xwinnmr 2.6. The 1D spectra were weighted with an exponential function using a 0.2 Hz line broadening factor. The 2D spectra were multiplied with a squared sine function shifted by $\pi/2$. In the indirect dimension, the number of data points was increased by a factor of two using forward linear prediction with 64 coefficients. The subtraction of 1D as well as of 2D spectra was performed using the supplied features within the Bruker software.

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