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Note

NMR Spectroscopy of ^{13}C-Enriched Polysaccharides: Application of ^{13}C–^{13}C TOCSY to Sugars of Different Configuration

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ABSTRACT: ^{13}C–^{13}C 2D TOCSY experiments were applied, with both carbon-13 and proton detection, to alleviate the problem of limited ^{1}H–^{1}H coherence transfer in certain sugars, e.g. those with the *manno* or *galacto* configuration. The ^{13}C–^{13}C couplings are not as dependent on the different sugar geometries and can be up to 50 times larger, i.e. $^{1}J_{CC}$ *vs.* $^{3}J_{HH}$. For bacterial polysaccharides, M_r *ca.* 10^4–10^5, loss of magnetization can be severe owing to long delays in ^{1}H–^{1}H TOCSY experiments, and these are circumvented by applying the spin lock on the ^{13}C nuclei. © 1998 John Wiley & Sons, Ltd.

KEYWORDS: NMR; ^{13}C NMR; TOCSY; saccharide; lipopolysaccharide

INTRODUCTION

In structural studies of bacterial polysaccharides by NMR spectroscopy great emphasis is put on the entangling of ^{1}H–^{1}H spin connectivity pathways. Once ^{1}H resonances have been assigned, ^{1}H–^{13}C, ^{1}H–^{31}P and/or ^{1}H–^{15}N correlations can be used to resolve ambiguous assignments and to obtain, in particular, sequence information between sugar residues in the polymer. Most often, a combination of ^{1}H–^{1}H DQFCOSY[1] and ^{1}H–^{1}H TOCSY[2] experiments is used to assign the ^{1}H NMR signals of saccharides. By choosing the mixing time of TOCSY experiments, all ^{1}H signals may be traced, starting from the anomeric protons. The efficiency of TOCSY transfers, however, is severely hindered when small vicinal ^{1}H–^{1}H spin couplings are involved (e.g. via H-4 of galactose or H-2 of mannose).

One-bond carbon–carbon coupling constants are of the order of 45 Hz[3–5] (in the present study, coupling constants were found in the range 35–55 Hz) and in common sugar residues the magnitude of $^{1}J_{CC}$ is not as geometry dependent as that of the three-bond proton–proton coupling constants. Hence TOCSY transfers in an experiment with the spin lock on the ^{13}C nuclei would not be as sensitive to the configuration of the sugar residue. Furthermore, the large $^{1}J_{CC}$ values should facilitate rapid coherence transfer, an advantage for large-sized molecules as polysaccharides (M_r 10^4–10^5), in which the use of long mixing times are not to be preferred owing to short T_2 relaxation times.

The use of ^{13}C-enriched samples has found many applications in the NMR spectroscopy of proteins[6] and of nucleic acids[7] and has facilitated the extension to larger structures that can be determined by NMR spectroscopy. For carbohydrates in general, only limited use has been made of ^{13}C-enriched samples. Investigations have been performed on compounds of low molecular weight, intermediate sized and high molecular weights. The NMR experiments employed have in the first case been, e.g., ^{13}C–^{13}C RELAY COSY on protected monosaccharides,[8] in the second case, e.g., HCCH-TOCSY,[9] 3D ^{1}H detected ^{13}C–^{13}C double quantum experiments[10] or 3D HOHAHA-HSQC and NOESY-HSQC experiments[11] on oligosaccharides and in the third case, e.g., ^{13}C–^{13}C COSY on the capsular polysaccharide from *Klebsiella* K3[12,13] or relaxation[14] and conformation[15] studies of the *Streptococcus mitis* J22 polysaccharide.

In this study, we applied ^{13}C–^{13}C 2D TOCSY experiments to the ^{13}C enriched O-antigenic polysaccharide isolated from *Escherichia coli* O25 in order to alleviate the problem of limited ^{1}H–^{1}H TOCSY coherence transfers in sugars commonly found in bacterial polysaccharides.

RESULTS AND DISCUSSION

Escherichia coli O25 was grown in a growth medium to which had been added uniformly ^{13}C-labeled D-glucose. After purification of the lipopolysaccharide (LPS) and subsequent delipidation, the polysaccharide (PS) was

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isolated by gel permeation chromatography. The structure of the O-antigenic PS from *E. coli* O25 has been reported previously[16] and consists of pentasaccharide repeating units with the following sugar residues: 6-deoxy-α-L-mannopyranose (α-L-Rha*p*), 2-acetamido-2,6-dideoxy-α-L-galactopyranose (α-L-Fuc*p*NAc), α-D-glucopyranose, β-D-glucopyranose and 2-acetamido-2,6-dideoxy-β-D-galactopyranose. In the first two sugar residues (Fig. 1), ^1H–^1H TOCSY transfer throughout the whole spin system is hampered owing to small proton–proton couplings ($^3J_{HH} < 2$ Hz).

In the ^1H NMR spectrum of the ^{13}C-enriched material, an overall ^{13}C enrichment of *ca.* 40% was estimated based on integration of the ^{13}C satellies. Matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectra[17] of labeled and unlabeled polysaccharide materials suggest that in the labeled PS ^{13}C enrichment has occurred for approximately half of the material and is randomly distributed throughout the polymer. In a ^{13}C–^{13}C DQFCOSY spectrum, however, analysis of cross peaks according to Jones and Sanders[12] showed that in sugar residues where labeling had occurred, all carbon positions were ^{13}C-enriched. That ^{13}C-enriched sugar residues are fully labeled is consistent with an uptake of D-[U-$^{13}C_6$]-glucose followed by the formation of glycosyl esters of nucleotide diphosphates, such as UDP-Glc, which may serve as a reservoir for the biosynthesis of other sugar residues.[18] Formation of the ^{13}C-labeled *N*-acetyl groups of the amino sugars was found to have taken place in the same way and to the same extent as in the sugar residues.

The residue-selective uniform ^{13}C enrichment made it possible to apply ^{13}C–^{13}C 2D TOCSY experiments to the polysaccharide. ^{13}C–^{13}C 2D TOCSY experiments with direct detection[19] were performed with spin lock times of 10 and 20 ms. Magnetization could be transferred from C-1, through the spin system, to C-5 in all sugar residues with a spin lock time of 20 ms (Figs 2 and 3). For some residues magnetization could be transferred throughout the whole spin system. Owing to the low sensitivity of the carbon-13 nuclei, the experimental times were long. The use of proton detected ^{13}C–^{13}C 2D TOCSY experiments, i.e. 2D planes of a 3D HCCH-TOCSY experiment,[20,21] (Fig. 4), drastically shortened the experimental time. With the longest spin lock time (23 ms) magnetization could be transferred from C-1,

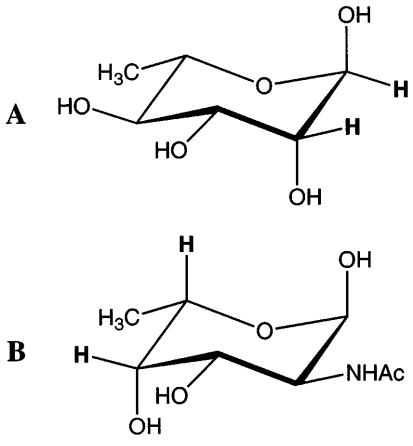

Figure 1. Two sugar residues, (A) α-L-Rha*p* and (B) α-L-Fuc*p*NAc, in which ^1H–^1H TOCSY transfer in hampered owing to small coupling constants. The protons with $^3J_{HH}$ less than 2 Hz are indicated in the two sugar residues.

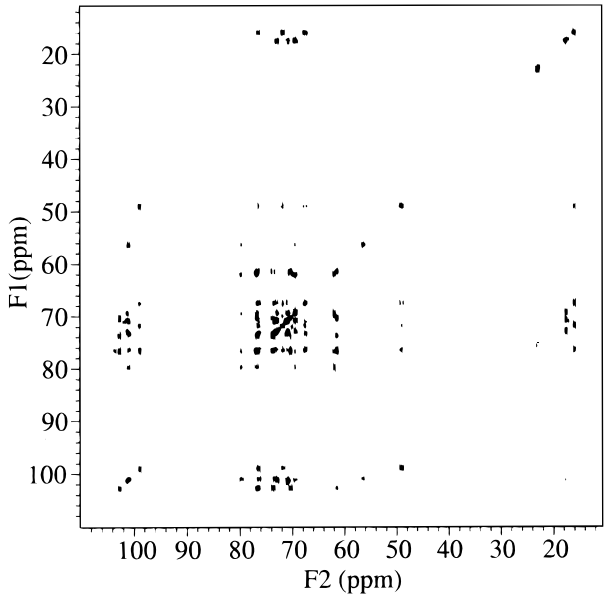

Figure 2. ^{13}C–^{13}C 2D TOCSY spectrum with carbon-13 detection of the *E. coli* O25 PS. Carbonyl signals have been aliased and the spin lock time was 20 ms.

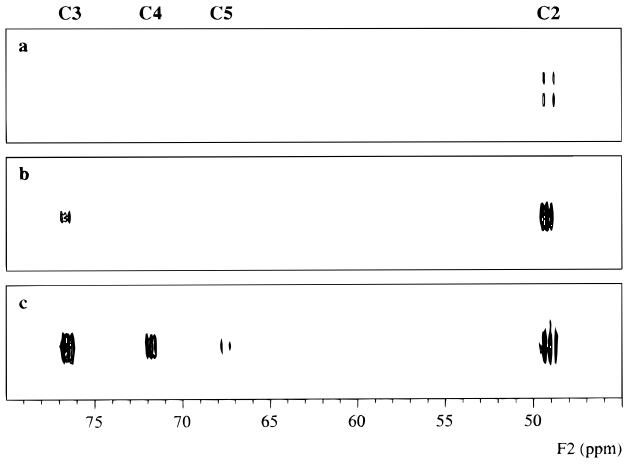

Figure 3. ^{13}C–^{13}C correlated spectra with carbon-13 detection of the *E. coli* O25 PS. Spectra have been expanded to show the chemical shift of the anomeric signal of the α-L-Fuc*p*NAc residue in f_1 and cross peaks from that signal in f_2. Assignments of signals are given above the spectra and the experiments performed were (a) ^{13}C–^{13}C DQFCOSY, (b) ^{13}C–^{13}C TOCSY with a spin lock time of 10 ms and (c) ^{13}C–^{13}C TOCSY with a spin lock time of 20 ms. The experimental times were about 20 h per experiment.

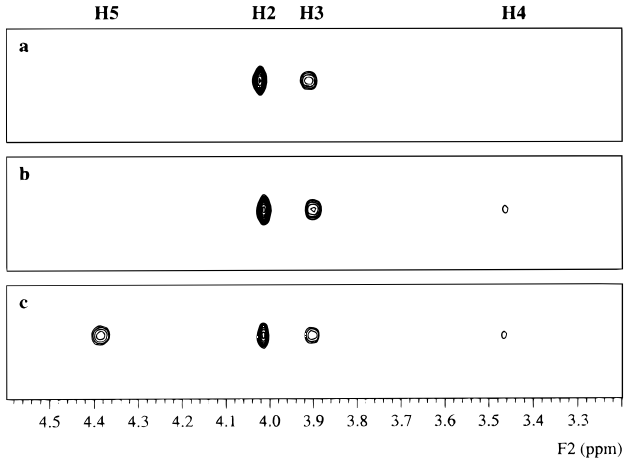

Figure 4. ^{13}C–^{13}C TOCSY spectra with proton detection of the *E. coli* O25 PS. Spectra have been expanded to show the chemical shift of the anomeric signal of the α-L-Rha*p* residue in f_1 and cross peaks from that signal in f_2. Assignments of signals are given above the spectra and the experiment performed was an f_1, f_3 2D plane of the 3D HCCH-TOCSY experiment using spin lock times of (a) 8, (b) 16 and (c) 23 ms. The experimental times were about 3 h per experiment.

through the spin system, to C-5 in most of the residues and in some residues through the entire spin system. A 3D HCCH-TOCSY experiment was also performed but, as reported earlier,[9] the resolution achieved with reasonable experimental time was insufficient to resolve overlapping signals in the carbon-13 dimension and did not yield any additional information. The assignments made using the ^{13}C–^{13}C TOCSY experiments were in agreement with those made on unlabeled material, using various standard 1D and 2D ^{1}H–^{1}H and ^{1}H–^{13}C correlated experiments.

Typically, ^{13}C resonances of saccharides appear between 60 and 110 ppm (7.5 kHz at 150 MHz). When carbonyl or carboxylic carbon atoms as well as methyl groups are involved, the ^{13}C chemical shift region is 160 ppm (24 kHz at 150 MHz). Longer spin lock times or higher r.f. power levels than those applied in this study would probably cause unacceptable sample heating. In the ^{13}C–^{13}C TOCSY experiment the r.f. power (in watts) needs to be increased by a factor of four in order to double the spectral width covered by the spin lock. Thus, at a lower magntic field, ^{13}C–^{13}C TOCSY experiments covering wider chemical shift range can be performed without serious sample heating. Both the spectral resolving power and sensitivity are, however, reduced.

As the repeating unit of the *E. coli* O25 PS contains sugar residues of both *manno* and *galacto* configuration TOCSY experiments with the spin lock on ^{13}C nuclei greatly facilitate signal assignment. Some polysaccharides, in particular capsular polysaccharides, have low solubility and high molecular weights and in these cases ^{13}C enrichment will be very valuable as these materials are amenable to ^{13}C–^{13}C TOCSY experiments. The present study shows that the use of ^{13}C–^{13}C 2D TOCSY experiments (with direct or indirect detection) overcomes the problem of limited coheence transfer in ^{1}H–^{1}H TOCSY experiments on sugar residues with small proton coupling constants.

EXPERIMENTAL

Bacterial growth and isolation of polysaccharide

Escherichia coli O25:11L was grown in TY-medium (Difco Laboratories, MI, USA) composed of tryptone (10 g) and yeast extract (5 g), supplemented with NH_4Cl (47 mM), Na_2HPO_4 (84 mM), KH_2PO_4 (44 mM) and Na_2SO_4 (1.6 mM) (Merck, Darmstadt, Germany). The TY-medium was further supplemented with 1 g of either D-glucose or uniformly ^{13}C enriched D-glucose, 98–99 atom% ^{13}C, 99%+ chemical purity (Cambridge Isotope Laboratories, Woburn, MA, USA). A solution of glucose in 10 ml of distilled water was sterilized by filtration and added to 3 l of TY-medium. Two precultures, 50 ml each of TY-medium, were incubated at 37 °C for 2 h, after which they were inoculated in the glucose containing TY-medium. After 4 h of incubation at 37 °C and constant aeration with sterile air, formalin [1% (*w/v*) final concentration] was added and the cultures were kept at 4 °C overnight. After centrifugation (8000g, 4 °C, 20 min) and subsequent washing with phosphate-buffered saline, the bacterial mass was suspended in distilled water and subjected to phenol–water extraction.[22] The yield of LPS was *ca.* 60 mg. Delipidation of the LPS (1% HOAc, 100 °C for 1 h) yielded, after gel permeation chromatography on a Bio-Gel P-2 column, *ca.* 10 mg of polysaccharide from each culture.

NMR spectroscopy

NMR spectra were recorded at 30 °C on a Varian Unity 600 spectrometer, equipped with a Varian 5 mm broadband probe, and a Varian Inova 600 spectrometer, equipped with a triple resonance PFG probe. Samples were dissolved in D_2O and internal TSP (sodium 3-trimethylsilylpropanoate-d_4, δ_H 0.0) or external acetone (δ_C 31.0) was used as a reference.

In the directly detected ^{13}C–^{13}C TOCSY experiment, a DIPSI-2[23] spin lock sequence was used. Spin lock times were 10 and 20 ms with $\gamma B_1/2\pi \approx 15$ kHz. WALTZ-16 ^{1}H broadband decoupling with $\gamma B_1/2\pi \approx 5.8$ kHz was used throughout all the ^{13}C detected experiments. A sweep width of 15 kHz was used in both dimensions and 96 transients of 2048 complex data points were accumulated for 256 increments. Data were zero filled to 2048 × 1024 points and treated with a

Gaussian apodization window in both dimensions prior to Fourier transformation. In the proton detected ^{13}C–^{13}C 2D TOCSY experiment, carbon-13 signals were aliased to reduce the carbon-13 sweep width to 10 kHz, while the proton sweep width was 2.2 kHz. A DIPSI-3[23] spin lock of 7.7 kHz was applied for 7.7, 15.5 and 23.3 ms in three different experiments. In the 3D HCCH-TOCSY experiment two transients with 512 complex data points were collected in the f_3 dimension. The number of points in the indirectly detected dimensions were 128 (f_1, proton) and 64 (f_2, carbon-13) and the sweep widths were the same as for the 2D planes. The spin lock of 7.7 kHz was applied for 15.5 ms. During the acquisition time in the proton detected experiments, a GARP broadband decoupling with $\gamma B_1/2\pi = 8.9$ kHz was used for ^{13}C decoupling.

Analysis of DQFCOSY cross peaks was performed according to Jones and Sanders.[12] Since all of the anti-phase cross peaks from the active couplings of the signals were split into in-phase doublets, due to passive coupling to a carbon-13 nucleus (except for C-1 and C-6), it was concluded that there were no ^{13}C–^{13}C–^{12}C combinations present. Hence, the ^{13}C enrichment had taken place uniformly within each labeled sugar residue. Examination of the intensity of the anomeric signals in the ^{13}C spectrum of the polysaccharide showed that enrichment had taken place to the same extent in all sugar residues.

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