Dipole-Dipole Cross-Correlation at ^{13}C Natural Abundance: A Structural Tool for Polysaccharides

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Carbohydrates are abundant and extremely diverse molecules including oligosaccharides, nucleotides, glycoconjugates, and polysaccharides. In the past few years numerous NMR methods have been developed for the study of oligosaccharides[1] and nucleotides[2] because of their combined biological relevance, high solubility, and the availability of ^{13}C-labeled samples. Polysaccharides represent the single most abundant source of carbohydrates and are important industrial products. These large molecules with sizes in the MDa range are seldom ^{13}C-labeled.[3] Structural studies of polysaccharides are focused on the determination of glycosidic linkages, the connections between the reducing end of one monosaccharide unit (the anomeric position) and the hydroxyl group of the neighboring monosaccharide unit (the aglyconic position). We present a NMR experiment allowing the determination of glycosidic linkages in unlabeled oligo- and polysaccharides using dipole−dipole cross-correlated relaxation.

Glycosidic linkages are typically determined by a combination of chemical analysis and two types of NMR experiments, namely HMBC[4] and NOESY.[5] Both experiments have serious drawbacks. The sensitivity of the HMBC experiment is very low since the magnetization flows from proton to carbon *and* back via weak long-range scalar couplings ($^3J_{CH} < 8$ Hz), times during which rapid transverse relaxation reduces signal intensities very effectively. The interpretation of polysaccharides NOESY spectra is very often hampered by serious overlap, by line-broadening stemming from the polysaccharides size, and from the presence of pervasive spin diffusion. These limitations make unambiguous structural determination very difficult.

In recent years, cross-correlated relaxation mechanisms have been used as structural tools and as probes for dynamics. In particular, cross-correlated relaxation rates were used for the determination of dihedral angles in labeled proteins,[6] to study protein dynamics[7] and for establishing sugar ring pucker modes in RNA.[8] Most of these experiments rely on relaxation mechanisms involving several heteronuclei, approaches that are clearly not applicable to natural abundance polysaccharides samples.

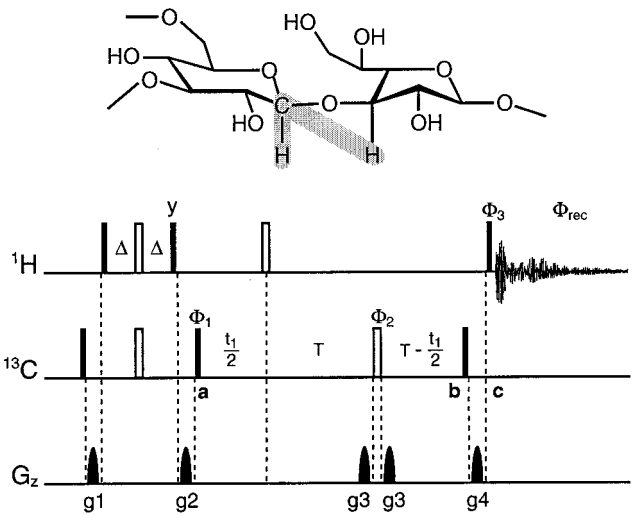

Figure 1. (Top) Schematic representation of two dipoles whose cross-correlation is measured in the experiment. (Bottom) Pulse sequence for the determination of glycosidic linkages in natural abundance ^{13}C carbohydrates by dipole-dipole cross-correlation. $\Delta = 1/[4(^1J_{CH})]$, $2T$ is the total cross-correlated relaxation time. $\phi_1 = x,-x$; $\phi_2 = 4(x),4(y),4(-x),4(-y)$; $\phi_3 = x,x,-x,-x$; and $\phi_{rec} = x,-x,-x,x,-x,x,x,-x$. TPPI was applied to ϕ_1. Gradients were: g1 = −51, g2 = 93, g3 = −17, and g4 = 23.

The experiment of Figure 1 uses cross-correlated relaxation between two dipoles centered on the same carbon atom, the C^1H^1/C^1H^n and C^nH^n/C^nH^1 dipole pairs, to transfer magnetization across the glycosidic bond (Figure 1, top). The sequence starts with INEPT that transforms proton magnetization into carbon coherence resulting in $2C_y{}^1H_z{}^1$ at point **a**. During the constant-time period $2T$, proton−carbon scalar couplings do not evolve, while carbon chemical shifts are modulated by t_1. Furthermore, cross-correlation between the C^1H^1/C^1H^n dipoles is not averaged leading to the two carbon antiphase terms, $2C_y{}^1H_z{}^1$ and $2C_y{}^1H_z{}^n$ (point **b**). At point **c** in the sequence, these two terms have been transferred into $2C_z{}^1H_y{}^1$ and $2C_z{}^1H_y{}^n$ that will be refocused through their respective scalar coupling during detection. The term $2C_z{}^1H_y{}^1$ refocuses through the large scalar coupling $^1J_{CH}$ and yields a direct peak at $(\omega_1, \omega_2) = (\Omega(C^1), \Omega(H^1))$, while the term $2C_z{}^1H_y{}^n$ is refocused through the small scalar coupling $^3J_{CH}$ and results in a cross-peak at $(\Omega(C^1), \Omega(H^n))$ characteristic of the glycosidic linkage between C^1 and C^n. The corresponding symmetrical peaks at $(\Omega(C^n), \Omega(H^n))$ and $(\Omega(C^n), \Omega(H^1))$ resulting from the cross-correlated relaxation between the C^nH^n/C^nH^1 dipoles provide confirmation of the glycosidic linkages.

Streptococcus thermophilus are lactic acid bacteria used in the (industrial) fermentation of milk into yogurts. In addition to releasing flavor molecules, bacteria secrete lactate, resulting in an acidification of the medium, and exopolysaccharides essential for the texture and consistency of the yogurt.[9] Lactic acid bacteria

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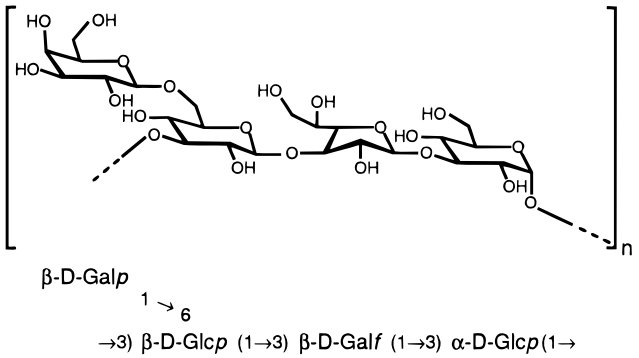

Figure 2. Structure of the repeating unit of the high molecular weight polysaccharide (>2 MDa, $n \sim 3{,}500$) secreted by *Streptococcus thermophilus* Sfi39.

strains secrete polysaccharides of widely differing structures studied in several laboratories.[10] One major aim is to correlate structures with rheological measurements. The cross-correlated dipole-dipole relaxation experiment of Figure 1 was applied to a sample of *S. thermophilus* Sfi39 polysaccharide whose structure was determined by NMR (Figure 2).[11] In Figure 3a, two symmetry-related peaks at $(\omega_1, \omega_2) = (\Omega(C3), \Omega(H1))$ and $(\Omega(C1), \Omega(H3))$ identify the glycosidic linkage β-D-Glc*p*-(1→3)-β-D-Gal*f*. Decoupled in the ω_1 dimension, the cross-peaks are antiphase in the ω_2 dimension with respect to the small long-range proton-carbon scalar couplings and in-phase with respect to proton-proton scalar couplings. The direct signals are clearly identified by their antiphase structure in the ω_2 dimension due to the one-bond proton-carbon scalar coupling ($^1J_{CH}$), whose value is a sensitive probe for the determination of the absolute (α or β) configuration of the corresponding monosaccharide.[12] Figure 3b shows all four expected cross-peaks linking the anomeric proton resonances with the aglyconic carbon chemical shifts in the Sfi39 polysaccharide.

The cross-correlated dipole-dipole relaxation experiment of Figure 1 can be more sensitive than HMBC in cases where transverse relaxation is fast. Cross-correlated dipole-dipole relaxation can create coherences across the glycosidic linkage more rapidly than the small proton-carbon scalar couplings. A constant-time period of $2T = 10$ ms is sufficient for substantial transfer mediated by cross-correlated relaxation, while periods of 30 to 80 ms are needed in the case of an HMBC. In the experiment of Figure 1, the diagonal peaks are detected with a sensitivity similar to HSQC, much higher than in HMBC where the transfer of direct peaks is either suppressed or left to evolve arbitrarily for the duration of the long transfer time while transverse relaxation reduces the magnetization. By acquisition of the experiment of Figure 1, it becomes unnecessary to acquire a ω_2-undecoupled HSQC for measuring anomeric one-bond proton−carbon scalar couplings necessary for determining the absolute conformation of the monosaccharide units.

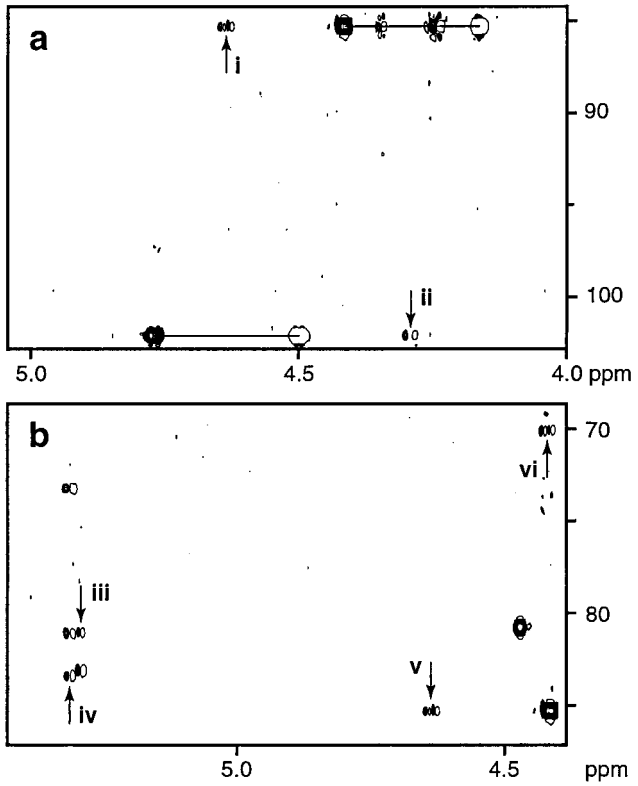

Figure 3. Constant-time (20 ms) cross-correlated dipole/dipole relaxation spectrum of the polysaccharide Sfi39 at natural ^{13}C abundance (67 °C, ^2H_2O) for the determination of glycosidic linkages. (a) Symmetry-related peaks (ω_1, ω_2) for the β-D-Glc*p*-(1→3)-β-D-Gal*f* glycosidic linkage: (i) β-D-Gal*f*(C3), β-D-Glc*p*(H1) and (ii) β-D-Glc*p*(C1), β-D-Gal*f*(H3). The full intensity diagonal signals are antiphase toward the one-bond proton-carbon scalar coupling ($^1J_{CH}$, solid line). (b) Four cross-peaks expected at $(\omega_1, \omega_2) = (\Omega(C^n), \Omega(H^1))$ corresponding to the four glycosidic linkages: (iii) α-D-Glc*p*(H)-(1→3)-β-D-Glc*p*(C), (iv) β-D-Gal*f*(H)-(1→3)-α-D-Glc*p*(C), (v) β-D-Glc*p*(H)-(1→3)-β-D-Gal*f*(C), and (vi) β-D-Gal*p*(H)-(1→6)-β-D-Glc*p*(C).

As cross-correlated relaxation rates are a function of the correlation times describing molecular tumbling,[8] NMR experiments based on these relaxation mechanisms are particularly suited for the study of very large molecular weight molecules such as polysaccharides. The combined use of different NMR experiments based on independent transfer mechanisms is a strong advantage in the determination of structural information, especially for the case of glycosidic linkages where the available information is sparse and often ambiguous. In conclusion, the application of dipole-dipole cross-correlated relaxation brings an additional tool for the structure determination of nonlabeled carbohydrates, especially suited for polysaccharides.

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