bovine serum (FBS), 100 U/mL penicillin, and 0.1 mg/mL streptomycin (Nacalai Tesque, Kyoto, Japan). Human colon carcinoma SW480 cells (American Type Culture Collection, Manassas, VA) and immortalized keratinocyte HaCaT cells (kindly provided by Dr. Tadashi Terui, Department of Dermatology, Nihon University School of Medicine) were cultured in DMEM containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Human ostesarcoma MG63 cells (RIKEN Cell Bank) were cultured in minimum essential medium containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. Human myeloid leukemia THP-1 cells and U937 cells (RIKEN Cell Bank) were cultured in RPMI1640 medium containing 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin. The condition of cell culturing was kept at 37 °C in a humidified atmosphere containing 5% CO_2.

Plasmids. The expression vectors pCMX-VDR, pCMX-RXRα, pCMX-VP16-VDR, pCMX-GAL4-RXRα, pCMX-GAL4-SRC-1, pCMX-GAL4-N-CoR, and pCMX-GAL4-SMART were reported previously.[13b] The nuclear receptor-interacting domains of SRC-1 (amino acids 595–771; GenBank accession no. U90661), N-CoR (amino acids 1990–2416; GenBank accession no. U35312), and SMRT (amino acids 2003–2517; GenBank accession no. AF113003) were inserted into the pCMX-GAL4 vector to make pCMX-GAL4-SRC-1, pCMX-GAL4-N-CoR, and pCMX-GAL4-SMRT, respectively. VDR responsive Spp × 3-tk-LUC and GAL4-responsive MH100(UAS) × 4-tk-LUC reporter vectors were previously reported.[14d] pGEX vector (GE Healthcare, Chalfont St. Giles, Buckinghamshire, UK) was used to generate glutathione transferase (GST) fusions.[30] The human recombinant VDR ligand-binding domain (LBD) (amino acids 140–427) was inserted into pGEX vector to generate pGEX-VDR.

Vitamin D Receptor-Binding Assay. The pGEX or pGEX-hVDR was expressed as a GST fusion protein in *Escherichia coli* BL21 (EMD Millipore). The cells were lysed by sonication in sonication buffer (50 mM Tris-HCl pH 8.0, 50 mM NaCl, 1 mM EDTA, and 1 mM DTT). Then 1 μg of the supernatants was diluted in binding buffer (25 mM Tris pH 7.5, 100 mM KCl, 25 mM DTT, 4 mM CHAPS, pH 7.5) containing bovine serum albumin (100 μg/mL). A solution containing an increasing amount of $1,25(OH)_2D_3$ (**1**) or synthetic analogues in 15 μL of EtOH was added to 570 μL of the receptor solution in each tube, and the mixture was vortexed 2–3 times. The mixture was incubated for 30 min at room temperature. [26,27-Methyl-^{3}H] $1,25(OH)_2D_3$ (PerkinElmer) in 15 μL of EtOH was added, vortexed 2–3 times, and the whole mixture was then allowed to stand at 4 °C for 20 h. At the end of the second incubation, 400 μL of dextran-coated charcoal solution (Sigma) was added to bind any free ligands (or to remove free ligands) and the sample was vortexed. After 30 min at room temperature, bound and free [^{3}H]-$1,25(OH)_2D_3$ were separated by centrifugation at 3000 rpm for 10 min at 0 °C. Aliquots (800 μL) of the supernatant were mixed with 9.2 mL of Bio Fluor (PerkinElmer) and submitted for radioactivity counting. Each assay was performed at least twice in triplicate.

Transcription Assays. HEK293 cells were cultured in Dulbecco's Modified Eagle's Medium containing 5% fetal bovine serum and antibiotic–antimycotics (Nacalai) at 37 °C in a humidified atmosphere of 5% CO_2 in air. Transfections of 50 ng of TK-Spp × 3-LUC reporter plasmid, 10 ng of pCMX-β-galactosidase, 15 ng of pCMX-VDR, and 15 ng of pCMX-RXRα for each well of a 96-well plate were performed by the calcium phosphate coprecipitation method as described previously.[14d] Then 18 h after transfection, test compounds were added. Cells were harvested after 16–24 h for luciferase and β-galactosidase activity using a luminometer (Molecular Devices, Sunnyvale, CA).

Mammalian two-hybrid assay for cofactor interaction to VDR was used 50 ng of TK-MH100(UAS) × 4-LUC reporter plasmid, 10 ng of pCMX-β-galactosidase, 15 ng of pCMX-GAL4-RXRα, pCMX-GAL4-SRC1, pCMX-GAL4-NCoR, or pCMX-GAL4-SMART and 15 ng of pCMX-VP16-VDR for each well of a 96-well plate. Luciferase data were normalized to the internal β-galactosidase control.

Reverse Transcription and Quantitative Real-Time PCR Analysis. For gene expression analysis, 1×10^4 cells per well were plated in 24 well plate. After 24 h, cells were treated with ethanol control or 100 nM of $1,25(OH)_2D_3$ or its analogues for 24 h. Total RNAs from samples were prepared by the acid guanidine thiocyanate–phenol/chloroform method.[31] cDNAs were synthesized using the ImProm-II reverse transcription system (Promega, Madison, WI).[14d] Real-time PCR was performed on the ABI PRISM 7000 sequence detection system (Applied Biosystems, Foster City, CA) using Power SYBR Green PCR Master Mix (Applied Biosystems). Primer sequences were as follows: CYP24A1:5′-TGAACGTTGGCTTCAGGAGAA-3′ and 5′-AGGGTGCCTGAGTGTAGCATCT-3′, TRPV6:5′-TGAACGTTGGCTTCAGGAGAA-3′ and 5′-AGGGTGCCTGAGTGTAGCATCT-3′, CAMP 5′-GCTAACCTC-TACCGCCTCCT-3′ and 5′-GGTCACTGTCCCCATACACC-3′, and ACTIN: 5′-GACAGGATGCAGAAGGAGAT-3′ and 5′-GAAG-CATTTGCGGTGGACGAT-3′. mRNA values were normalized to an amount of ACTIN mRNA.

Protein Expression and Purification. The rat VDR LBD (residues 116–423, Δ165–211) was cloned as an N-terminal His6-tagged fusion protein into the pET14b expression vector and was overexpressed in *E. coli* C41. The cells were grown at 37 °C in LB medium (including 100 mg/L ampicilin) and were subsequently induced for 6 h with 15 μM isopropyl-β-D-thiogalactopyranoside (IPTG) at 23 °C. The purification procedure included affinity chromatography on a Ni-NTA column, followed by dialysis and ion-exchange chromatography (SP-Sepharose). After tag removal by thrombin digestion, the protease was removed by filtration through a HiTrap benzamidine column, and the protein was further purified by gel filtration on a Superdex 200 column. The purity and homogeneity of the rVDR LBP were assessed by SDS-PAGE.

Crystallization. Purified rVDR-LBD solution was concentrated to about 0.75 mg/mL by ultrafiltration. To an aliquot (800 μL) of the protein solution was added a ligand (**4a**, **4b**, **5a** or **5b**, ca. 10 equiv); the solution was further concentrated to about 1/8, and then a solution (25 mM Tris-HCl, pH 8.0; 50 mM NaCl; 10 mM DTT; 0.02% NaN3) of coactivator peptide (H2N-KNHPMLMNLLKDN-CONH2) derived from DRIP205 was added. This solution of VDR/ligand/peptide was allowed to crystallize by the vapor-diffusion method that used a series of precipitant solutions containing 0.1 M MOPS-NaOH (pH 7.0), 0.1–0.4 M sodium formate, 12–22% (w/v) PEG4000, and 5% (v/v) ethylene glycol. Droplets for crystallization were prepared by mixing 2 μL of complex solution and 1 μL of precipitant solution, and droplets were equilibrated against 500 μL of precipitant solution at 20 °C. It took 1–2 days to obtain crystals of X-ray diffraction quality for VDR complexes with **4a**, **4b**, **5a**, or **5b** as a ligand.

Diffraction Experiment and Structure Analysis. Prior to the diffraction data collection, crystals were soaked in a cryoprotectant solution that contained 0.1 M MOPS-NaOH (pH 7.0), 0.1–0.4 M sodium formate, 15–20% PEG4000, and 17–20% ethylene glycol. Diffraction data sets were collected at 100 K in a stream of nitrogen gas at beamlines BL-6A of KEK-PF and NW12A of PF-AR (Tsukuba, Japan). Reflections were recorded with an oscillation range per image of 1.0°. Diffraction data were indexed, integrated, and scaled using the program HKL2000.[32] The structures of the complex were solved by molecular replacement with the program CNS,[33] and finalized sets of atomic coordinates were obtained after iterative rounds of model modification with the program XtalView[34] and after refinement with CNS by rigid body refinement, simulated annealing, positional minimization, water molecule identification, and individual isotropic B-value refinement.

■ ASSOCIATED CONTENT

Supporting Information

The X-ray crystal structure of rVDR-LBD complexed with ADTK2 (**4a**); unit cell structures of the ternary rVDR-LBD complexes with **4b** (ADTK1), **5a** (ADTK3 or $1,25(OH)_2D_3$ (**1**), and DRIP205; inter unit-cell interactions in the rVDR-LBD complex with **4b**; ternary rVDR-LBD complexes with ligands (**4b**, **5a**, and **5b**) and DRIP205 peptide in canonical active conformations; summary of data collection statistics and refinement; X-ray crystal data of compound **4a** (ADTK2). This material is available free of charge via the Internet at http://pubs.acs.org.

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Accession Codes

PDB codes for **4b**, **5a**, and **5b** are 3VTB, 3VTC and 3VTD, respectively.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

DMP, dimethylaminopyridine; CSA, camphor sulfonic acid; DMP, Dess–Martin periodinane; CBS, Corey–Bakshi–Shibata; RXRα, retinoid X receptor α; SRC-1, steroid receptor coactivator 1; NCoA1, nuclear receptor coactivator 1; N-CoR, nuclear receptor corepressor 1; SMRT, silencing mediator of retinoic acid and thyroid hormone receptor; N-CoR2, nuclear receptor corepressor 2; AF2, activation function 2; *CAMP*, cathelicidin antimicrobial peptides; *TRPV6*, transient receptor potential vanilloid 6; MTPA, α-methoxy-α-(trifluoromethyl)]-phenylacetyl; BMTO, B-methyl-4,5,5-triphenyl-1,3,2-oxazaborolidine; BMS, borane–dimethyl sulfide complex

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Indirect Detection of Hydroxy Proton Exchange Through Deuterium-Induced ^{13}C-NMR Isotope Shifts

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Contents

Abstract

Hydroxy groups are abundant on glycans and stabilize interactions with binding partners by forming intra- and inter-hydrogen bond networks. Information on the hydroxy proton and its bonding could be very useful for understanding glycan structure, their interactions, and their dynamics. Unfortunately, hydroxy protons are overlooked in NMR analyses because samples are typically measured in D_2O. Furthermore, their rapid exchange with water protons makes direct NMR detection difficult. We have developed a dynamic ^{13}C-NMR technique employing deuterium-induced ^{13}C-NMR isotope shifts, which provides indirect observation of exchangeable hydroxy protons through neighboring ^{13}C signals. In the presence of both H_2O and D_2O and a slow exchange environment, a doublet of each isotopomer,

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^{13}C-OH and ^{13}C-OD, is observed. A fast exchange environment makes each isotopomer indistinguishable and a sharp single signal appears at the average chemical shift. Thus, the ^{13}C-line shape is highly dependent on the OH/D exchange rate, which, in turn, reflects the presence/absence/strength of hydrogen bonding. This simple ^{13}C-NMR strategy is suitable for analyzing extremely weak interactions including bonds between carbohydrates and those involved in metal coordination.

Keywords
^{13}C-NMR • Isotope shift • H/D exchange • Hydroxy group • Hydrogen bond • β-glucan • LewisX • Degree of polymerization

Introduction

Glycans participate in biological interactions by forming intricate networks of hydrogen bonds using their hydroxy functionalities. Hydrogen bonds can be likened to glue to precisely attach carbohydrates to proteins and carbohydrates to carbohydrates. Hydrogen bonds within carbohydrates limit orientation and conformation of the sugar residues. Thus, hydroxy groups are extremely important in understanding glycan structure and function.

Unfortunately, ^{1}H-NMR spectra of the extremely hydrophilic glycans are usually collected in D_2O, which precludes the observation of hydroxy protons. Even when ^{1}H-NMR spectra are collected in H_2O, the rapid exchange of sugar hydroxy protons hampers direct observation of meaningful signals. The use of low temperature, set just above the freezing point of the aqueous solution, does improve signals from hydroxy protons; however, broadening and overlap still make detailed NMR analysis difficult. Currently, detection of hydroxy protons in aqueous media requires either low temperature with coexisting organic solvent (10–15 % acetone or methanol) or utilization of a capillary NMR tube to counter freezing. The use of organic solvents is best avoided as they often perturb proton exchange rates and can in fact abolish binding of a glycan to a cognate protein partner (Siebert et al. 2000).

Although several NMR parameters can potentially characterize the behavior of hydroxy groups, here we focus on the rate of exchange with water as it is extremely helpful in identifying hydrogen bonds, especially the weak ones involving carbohydrates. Exchange rates are ranging from 1 to 60 s^{-1} (Siebert et al. 2003), and such a wide range clearly indicates that this parameter is useful for detecting hydrogen bonds. This chapter describes a ^{13}C-NMR method for measuring proton exchange at hydroxy groups in water (Hanashima et al. 2011, 2014).

Principles

We have observed deuterium secondary isotope shifts on ^{13}C-chemical shifts (deuterium-induced ^{13}C-NMR isotope shift) at the geminal (two bonds, H-O-C) ^{13}C-signal of the exchangeable hydroxy proton (Fig. 1). Deuterium-induced

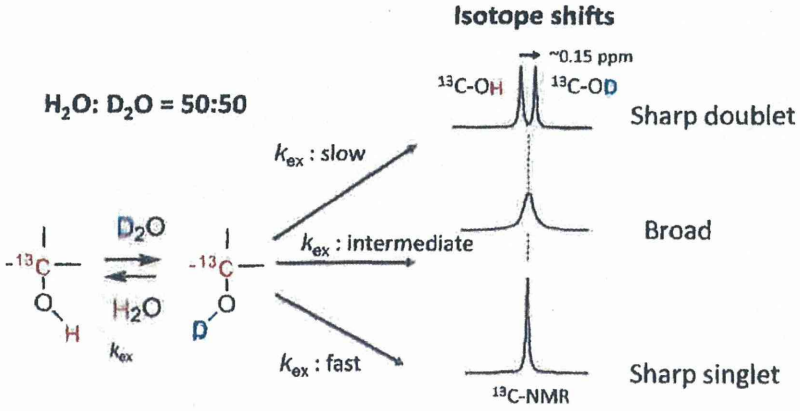

Fig. 1 Deuterium-induced ^{13}C-NMR isotope shifts for analysis of the hydroxy proton exchange rate. A glycan is dissolved in water consisting of H_2O/D_2O = 50:50. When the exchange rate k_{ex} is fast, a sharp singlet is detected; when slow, a doublet originating from ^{13}C-OD and ^{13}C-OH is observed (Adapted from Hanashima et al. (2014) with slight modifications with permission of the Royal Society of Chemistry)

^{13}C-NMR isotope shifts (DIS) on a series of mono- and disaccharides were initially reported using a dual coaxial NMR tube with separated cells (Pfeffer et al. 1979). In H_2O and D_2O mixtures, the line shape of the ^{13}C-OH/^{13}C-OD signal is highly dependent on the H/D exchange rate. During slow H/D exchange, DIS are observed for corresponding isotopomers, ^{13}C-OH and ^{13}C-OD with 0.09–0.15 ppm difference, whereas for fast exchange, the equilibrium makes each isotopomer indistinguishable and a sharp singlet signal is obtained at an averaged chemical shift. In the intermediate exchange range (k_{ex} ~ 10–20 s^{-1}), a broad signal appears at an averaged chemical shift. These properties allow the ^{13}C-NMR line shape to be used as an indicator of a hydrogen bond.

The coalescence temperature of the ^{13}C signals is simply determined by collecting the NMR data at various temperatures. The hydroxy-proton exchange rate at the coalescence temperature (Tc) is estimated by applying the Gutowski equation:

$$k_{ex} = \pi(\Delta\nu)/2$$

in which k_{ex} is the proton exchange rate (s^{-1}) at T_c (K) and $\Delta\nu$ is the DIS at the temperature (Hz).

Experimental Procedures

The samples were dissolved in 10–20 mM sodium acetate buffer, pH 6.0, with an H_2O/D_2O ratio (v/v) as in A, B, or C:

(A) 10 % D_2O/90 % H_2O
(B) 50 % D_2O/50 % D_2O
(C) 90 % D_2O/10 % H_2O (or 100 % D_2O)

It should be noted that exchange rates are extremely sensitive to pH and ionic contaminants. One-dimensional ^{13}C-NMR spectra were obtained, for example, from 263 to 298 K in 5 K increments. To avoid freezing, an NMR tube with a small diameter (1–3 mm) can be used. The ^{13}C chemical shift is given with reference to a chemical shift standard such as 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS) at 0 ppm. The chemical shift of the standard should be insensitive to the solvent (H_2O and D_2O). Digital resolution of the ^{13}C-NMR spectra needs to be typically 1–2 Hz/point in order to read the DIS accurately. The DIS could be in the range of 10–20 Hz at the ^{13}C observation frequency of 150 MHz (corresponding to ^{1}H frequency of 600 MHz). The DIS is shown in ppm, which is independent of the observation frequency. However, the coalescence temperature (T_c) will vary depending on the observation frequency according to the Gutowski equation.

Results

Two examples suffice to illustrate the application of DIS for the analysis of 3D structure and carbohydrate interactions.

Structure Formation of Short β-Glucan Chain

β1,3-Glucans, abundant in fungi, have the potential to activate an immune response against various pathogens (Goodridge et al. 2009). Part of the action is exerted through the C-type lectin-like receptor Dectin-1, but details of the interaction mechanism, especially with respect to glucan chain length, have been a matter of debate (Palma et al. 2006). To analyze the structure of β1,3-glucans, we have focused on proton exchanges of the hydroxy groups of short glucans with different chain lengths (degree of polymerization, DP) (Hanashima et al. 2014). ^{13}C-NMR on DP7 and laminarin (average DP, 25) were performed in 10 mM sodium acetate buffer (pH 6.0) composed of 50 % H_2O/50 % D_2O. Figure 2 shows that the C2 and C4 signals both carry secondary hydroxy groups. We find that there is no significant difference in the signal patterns at C2 of DP7 (red signal) and laminarin (black signal) at 5 °C (left panel in Fig. 2). The signals at 76.2 ppm gave sets of ^{13}C-OH/D isotopomer doublets with 0.06 ppm difference. The DIS doublets all coalesced on raising the temperature from 5 °C to 10 °C. The result apparently indicates that the ^{13}C-OH/D exchange rates at the C2 hydroxy group are very similar. The ^{13}C-NMR signal at C4 is in sharp contrast (right panel in Fig. 2). When the experiment was performed at 5 °C, the C4 signals at 70.9 ppm gave broad coalesced signals for DP7 (black signal) and a set of ^{13}C-OH/D isotopomer doublet signals 0.07 ppm apart for laminarin (red signal). The DIS doublet of laminarin coalesced at 15–20 °C. Evidently, exchange of ^{13}C-OH/D at C4 of laminarin is significantly slower than that of DP7. This is likely due to

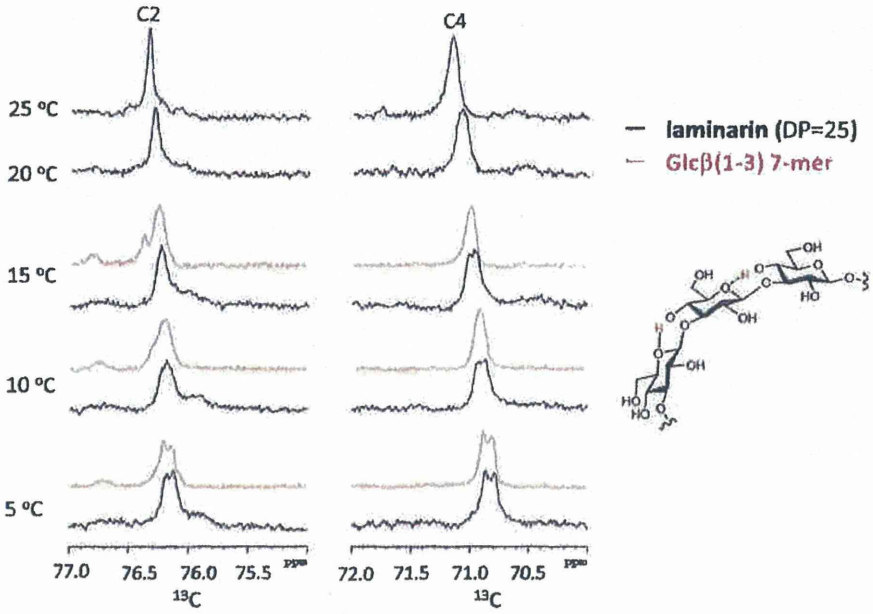

Fig. 2 125 MHz ^{13}C-NMR spectra of β1,3-glucans DP7 (*red signal*) and laminarin (DP ~ 25; *black signal*) at 5, 10, 15, and 20 °C. ^{13}C-NMR signals of C2 (*left*) and C4 (*right*) are shown. Ligands were dissolved at 10 mg/mL in 10 mM sodium acetate buffer (pH 6.0) composed of H_2O: D_2O = 50:50

the C4-OH of laminarin forming a hydrogen bond to either O5 or O6 of the nascent Glc residue.

LewisX-LewisX Interaction

LewisX antigen consisting of a trisaccharide of galactose (Gal), fucose (Fuc), and *N*-acetylglucosamine (GlcNAc) occurs at the termini of glycolipids and glycoproteins on cellular surfaces (Fig. 3a). The structure initiates cell-cell interaction by forming a LewisX-LewisX complex in a Ca^{2+}-dependent manner (Eggens et al. 1989). We have investigated the LewisX interaction by focusing on the fast exchange of OH groups because of the expectation that they are involved directly in stabilizing tertiary structures through hydrogen bonding and/or coordinating metal ions (Hanashima et al. 2011).

^{13}C-NMR spectra were collected at various temperatures from −10 °C to +20 °C. Again a 3-mm diameter NMR tube was employed to avoid freezing. In the presence of 1.0 M Ca^{2+} and at 5 °C, doublets originating from Fuc2, Fuc4, Gal2, and Gal6 carbons were observed (Fig. 3c), whereas none were forthcoming in the absence of Ca^{2+} (Fig. 3b). It clearly indicates that proton exchange is significantly slowed in

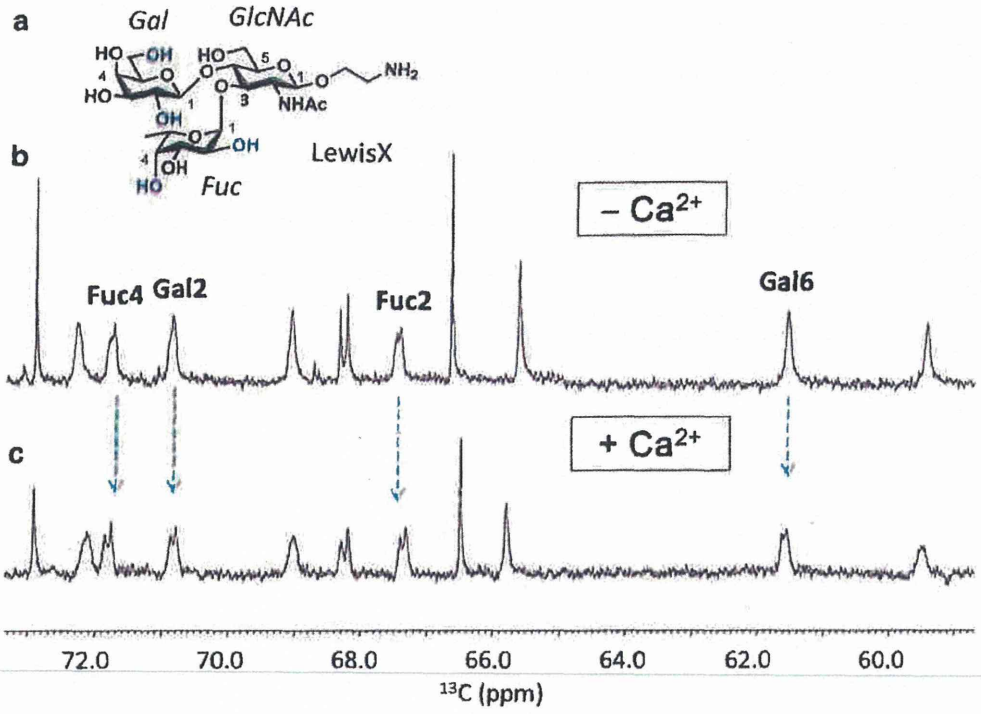

Fig. 3 225 MHz ^{13}C-NMR spectra of 40 mM LewisX with and without 1.0 M $CaCl_2$ at 5 °C. 10 mg trisaccharide was dissolved in 10 mM sodium acetate buffer, pH 6.0 in H_2O:D_2O = 1:1 (v/v). (**a**) Chemical structure of LewisX, (**b**) ^{13}C-NMR spectrum of LewisX without Ca^{2+}, (**c**) ^{13}C-NMR spectrum of LewisX with 1.0 M Ca^{2+} (Adapted from Hanashima et al. (2011) with slight modifications with permission of the Royal Society of Chemistry)

the Ca^{2+}-mediated LewisX interaction. Stabilization of these hydroxy protons suggests that they play a significant role in LewisX-Ca^{2+} complex formation through the formation of inter- and intramolecular hydrogen-bond networks with neighboring hydroxy groups.

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