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Paramagnetic Lanthanide Tagging for NMR Conformational Analyses of N-Linked Oligosaccharides

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It is estimated that more than half of all proteins in nature are post-translationally modified with various oligosaccharides.[1] The oligosaccharides are critical for biomolecular recognition events that mediates cell–cell communication and viral infections. N-linked oligosaccharides form a major class of the glycoprotein glycans, which commonly share the innermost *N,N′*-diacetylchitobiose structure covalently attached to the amide group of the asparaginyl side chains. Accumulating evidence[2] indicates that the N-linked oligosaccharides not only regulate biological functions of extracellular or cell-surface proteins but also serve as tags of proteins, determining their fates in cells, that is, folding, translocation, and degradation. The biological codes carried by the N-linked oligosaccharides are expressed as specific conformations recognized or selected by the carbohydrate-binding proteins collectively termed lectins.[2] Recent progress in glycomics has made it possible to profile N-linked oligosaccharides and determine their sequences, linkages, and positions on proteins.[3] However, conformational characterization of the individual oligosaccharides remains a challenging task because the flexible properties preclude X-ray crystallographic approaches. Although NMR spectroscopy has great potential to provide information on structure and dynamics of oligosaccharides,[4] the applicability of the NOE-based approach, widely used for protein-structure determination, is limited by the insufficiency of distance-restraint information as a consequence of the low proton density in oligosaccharides and the exceedingly low number of proton–proton NOEs that restrain interglycosidic linkages. Hence, it is highly desirable to develop NOE-independent approaches for determining the oligosaccharide conformations and dynamics.

Paramagnetic effects, such as pseudocontact shifts (PCSs) induced by lanthanide ions with an anisotropic magnetic-susceptibility tensor ($\Delta\chi$ tensor), offer long-distance information on conformations and dynamics of biological macromolecules.[5] Indeed, over the last few years, several lines of NMR studies have been reported by using PCSs as conformational restraints of proteins.[6] Herein we present an application of paramagnetic effects to characterize the carbohydrate conformations by using new lanthanide tags attached to the reducing end of an N-linked oligosaccharide. To develop a general method, we focused on the common core structure shared among all N-linked oligosaccharides, that is, *N,N′*-diacetylchitobiose. We focused on the induced PCSs of the CH groups of this disaccharide and extracted unique information on the glycosidic-linkage conformation.

The modified disaccharide **1** was synthesized as shown in Scheme 1. An ethylenediaminetetraacetic acid (EDTA) derivative was designed to serve as the paramagnetic tag by chelation to a lanthanide ion. A rigid phenylene spacer was inserted to suppress unfavorable relaxation enhancement of the carbohydrate resonances originating from the protons spatially proximal to the coordinated paramagnetic metal ion. The rigidity of the tag as well as the stability of the lanthanide complex are crucial factors for unambiguous interpretation of the PCS data. By selective amination and subsequent acylation reactions, *N,N′*-diacetylchitobiose was attached to this EDTA derivative through an amide linkage that mimics the "N-linked" oligosaccharides.

^{1}H NMR spectral changes of an aqueous solution of **1** (3 mM) were observed upon titration with paramagnetic Tm^{3+}. The addition of up to one molar equivalent of the ion generated a new set of peaks originating from **1** with concomitant disappearance of the original peaks. No further chemical-shift changes were induced with excess amounts of the lanthanide ion. These observations indicate that the lanthanide ion is bound at the specific site of **1** in a slow exchange regime; this gives rise to a stable 1:1 complex. By

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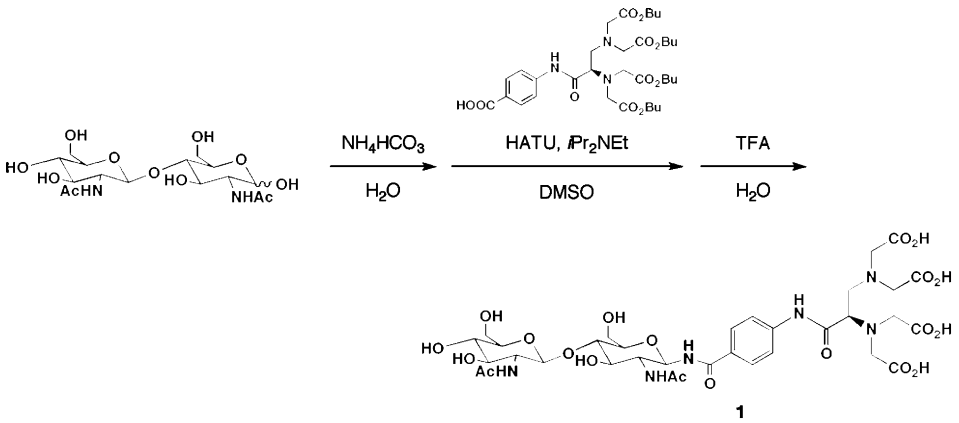

Scheme 1. Introduction of the lanthanide-chelating unit to *N,N′*-diacetylchitobiose (HATU = 2-(7-aza-1*H*-benzotriazole-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate, TFA = trifluoroacetic acid).

^{1}H–^{13}C HSQC experiments, the PCS values were measured as the differences of ^{1}H and ^{13}C chemical shifts compared with the compound chelated to the diamagnetic La^{3+} ion (Figure 1). The observed PCS values were largest (1.1 ppm)

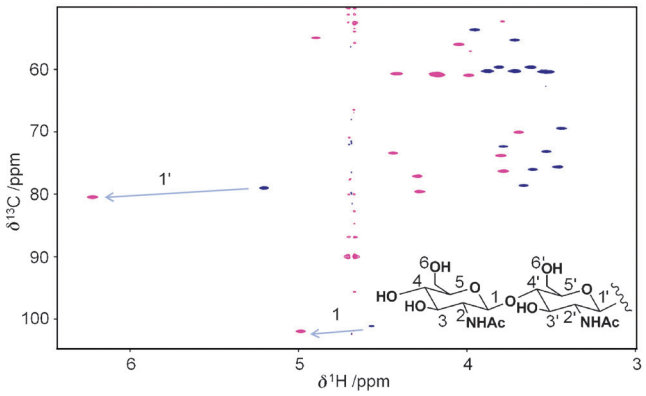

Figure 1. ^{1}H–^{13}C HSQC spectra of **1** complexed with Tm^{3+} (red) and La^{3+} (blue). The chemical-shift perturbations of the anomeric CH groups are indicated by arrows.

for C1, the anomeric carbon located at the reducing terminus, and smaller for the more distal atoms (Table S1 in the Supporting Information).

For quantitative validation of our approach, the experimentally obtained PCS values were compared with those calculated from the 3D model of **1**, which was built based on a reported conformation of *N,N′*-diacetylchitobiose (with the torsional angles defined by O5-C1-O4′-C4′ and C1-O4′-C4′-C5′ of −56 and −106°, respectively).[7] The components of the $\Delta\chi$ tensor were determined for this model by employing the experimental PCSs. The value of these components, $\Delta\chi_{ax}$ and $\Delta\chi_{rh}$, were estimated to be -7.66×10^{-32} and -2.29×10^{-32} m^3. As shown in Figure 2, the back-calculated PCS values are in excellent agreement with the experimental data ($Q = 0.03$).[8] Likewise, excellent agreement, with Q values of 0.02, 0.05, and 0.07, was observed by using other lanthanide ions, Ho^{3+}, Er^{3+}, and Yb^{3+} (see the Supporting Information). These results indicate that the common innermost part of the N-linked oligosaccharides exhibits a rigid conformation, which is little affected by the attachment of a tag. The conformational rigidity of the glycosidic linkage of this disaccharide agrees with results from molecular-dynamics simulations during 10 ns (data not shown). The behavior of *N,N′*-diacetylchitobiose is thus different from that of lactose investigated with the same approach.[9]

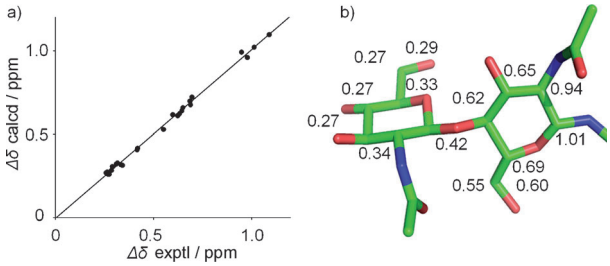

Figure 2. Analysis of the PCS data: a) Correlation between the experimentally observed and back-calculated PCS values. b) The 3D model of the carbohydrate moiety of **1**. The observed PCS values (ppm) of each proton are inserted. Determination of the $\Delta\chi$ tensor and back-calculation were performed with a modified version of MSpin.[11]

In conclusion, we have demonstrated the utility of the lanthanide tagging method, which provides valuable information on carbohydrate conformations in solution. Especially, the success of introducing a tag at the reducing-terminal, rigid disaccharide will open up a new avenue for NMR characterization of conformations, dynamics, and interactions with lectins of a variety of the N-linked oligosaccharides, including high-mannose-type oligosaccharides involved in the glycoprotein-fate determination in cells. To deal with these larger, N-linked oligosaccharides, the lanthanide ions with larger $\Delta\chi$ components, such as Dy^{3+} and Tb^{3+} would need to be used as sources of long-distance information. It is also expected that PCSs induced by lanthanide tagging contribute to peak separation in the highly degenerate spectra of the larger oligosaccharides. The applicability of our approach will be strengthened by combining it with stable isotope labeling of the oligosaccharides.[10] This line of studies is underway in our laboratories.

Experimental Section

All NMR spectra were recorded on JEOL JNM ECA-600 spectrometer equipped with a 5 mm FG/HCN probe. For PCS observation, ^{1}H–^{13}C

HSQC spectra were recorded at 300 K with 512 (t_1) and 1024 (t_2) complex points. NMR spectra were processed and analyzed with the programs NMRPipe[12] and Sparky.[13]

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Keywords: conformation analysis · lanthanides · NMR spectroscopy · oligosaccharides · paramagnetism

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