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NMR Study on a Novel Mucin from Jellyfish in Natural Abundance, Qniumucin from *Aurelia aurita*

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A novel mucin (qniumucin), which we recently discovered in jellyfish, was investigated by several NMR techniques. Almost all the peaks in the ^{13}C and proton NMR spectra were satisfactorily assigned to the amino acids in the main chain and to the bridging GalNAc, the major sugar in the saccharide branches. The amino acid sequence in the tandem repeat part (−VVETTAAP−) was reconfirmed by the cross-peaks between alpha protons and carbonyl carbons in the HMBC spectrum. A connectivity analysis around the *O*-glycoside bond (GalNAc−Thr) was also performed, and detailed information on the local configuration was obtained by the DPFGSE-NOE-HSD technique. The strategy and the results described in this paper can be extended to the structural analysis of general *O*-glycan chains, which are more complex than the present mucin. NMR analyses reveal the simple structure of qniumucin extracted by the present protocol, and the homogeneity and purity of qniumucin are probably the result of it being extracted from jellyfish, a primitive animal.

Mucin is a family of glycoproteins found in almost all animals as the main components of mucus, which is indispensable for sustaining life.[1−11] It has a wide variety of functions, including acting as a moisture holder, an antimicrobial, a counterpart of lectins, an adsorbent, and a surfactant. Mucin or "mucin domain" is defined as a polymer or a partial structure composed of a single protein main chain with branches of oligosaccharides connected at serine (Ser) or threonine (Thr) residues by *O*-glycoside bonds.

Although mucin itself is normally found as an extracellular substance, its complex structure has not yet been artificially reproduced by synthesis even when the main chain sequence of amino acids has been identified from genes because *O*-glycosylation occurs as a complex post-translational modification, which is an interplay of numerous enzymes and organs (such as the Golgi apparatus) in cellular systems. Therefore, the extraction of mucin from animals or plants in natural abundance is the sole effective procedure at present to provide mucins as mass producible materials for commerce.

Mucins extracted from domestic animals are commonly used as chemical reagents or food additives. They are divided into two categories, submaxillary mucins and gastric mucins. Those of the former group, i.e., bovine, ovine, and porcine submaxillary mucins (BSM, OSM, and PSM), have relatively high homogeneity with a simple main chain and simple glycochains, while those of the latter group are provided as mixtures of unresolved composition.

Most mucins have repeating partial sequences of amino acids involved in the peptide main chain, which is called the "tandem repeat" with various repeating periods. For human mucins, more than 20 of which are known, MUC5AC, for example, has a short period of 8 residues, while MUC6 has a long one of 169 residues. Although these tandem repeats are identified on human DNAs, most natural mucins discovered previously possess multiple components in the branching saccharides (glycoform) and extra domains different from the tandem repeat at both ends or in the middle of the protein chain. This structural complexity has prevented the study of each mucin as a well-defined single substance. Therefore, while studies of the extraction of mucins from various natural sources have a long history in biological science, structural investigations at the molecular level have not yet been performed extensively.

NMR is expected to bring a considerable advantage to the structural analysis of mucins[12−20] because the connectivity of the oligosaccharide and the nature of each monosaccharide can be determined without ambiguity, as has been confirmed for small moieties.[14] For intact mucins in natural abundance, however, only a few results have been presented to date, probably because of the above-mentioned inhomogeneous property of mucins as large macromolecules. Isotope concentration techniques are also difficult to apply to natural mucins. One exception is OSM, which has been extensively studied in natural abundance by NMR owing to its relatively simple structure with short glycochains.[15−19]

We have recently extracted a novel mucin named qniumucin (Q-mucin) from jellyfish,[21] whose total structure of the tandem repeat part is shown in Scheme 1. Although the analysis of the branched glycoside chain was not completed because of the existence of glycoforms, no extra protein sequence was recognized other than this tandem repeat following amino acid analysis. Q-mucin, the structure of which is as simple as that of OSM, has the potential to be an inexpensive mass-producible chemical resource in contrast to OSM because extremely high amounts of jellyfish can be collected from the ocean as marine wastes accumulated at power plants and fisheries. We expect that it can also be used as a starting material for synthesizing designer mucins employing transglycosidases,[22] and therefore, NMR analyses will be effective in supporting this technology. The tandem repeat of Q-mucin is composed of eight amino acid residues of five types, −VVETTAAP− with a small amount of −VIETTAAP−. Its N-terminus is open and no extra domains are detected by either content or sequence analysis of amino acids.

In our previous paper,[21] which was based on a simple composition analysis of monosaccharides, the branched saccharide parts connecting two Thr's in the tandem repeat were found to be simple, and most of them were speculated to be −GalNAc (*N*-acetyl galactosamine) or −GalNAc-Gal (galactose). However, a varied composition of monosaccharides is detected after fractionation by ion-exchange HPLC, thereby showing the existence of glycoforms. The composition changes with each extraction even from the same species. Moreover, one unidentified monosaccharide component, referred as "X" in this paper, was observed as a major component.

To clarify the composition and structure of the branched glycoside chains, further investigation was considered indispensable. In this paper, we present new results obtained from the NMR analysis of Q-mucin without any isotope labeling or concentration

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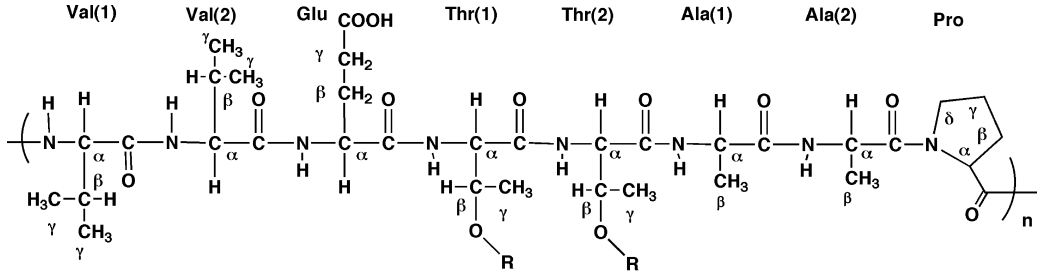
Scheme 1. Structures of "Tandem Repeat" Part of Qniumucin Expressed as R = GalNAc or X, $n > 1$[a]



[a] The model peptide used in this study is also expressed as R = H and $n = 1$.

Table 1. Monosaccharide Composition of the Sample Used in This Study

monosaccharide	composition (pmol)	molar ratio
GalNAc	27.82	1.0
X (unidentified)[a]	10.01[a]	0.4
Ara	4.54	0.2
Gal	3.09	0.1

[a] The amount of unidentified component (X) is estimated assuming that its signal to mol ratio in a fluorescence detector on HPLC is identical to that of GalNAc.

technique, which is difficult to apply to raw materials obtained from nature. This study indicates the possibility of NMR analysis on glycoproteins including mucins as extracted and purified from natural resources. The results obtained from this simple but typical mucin will provide useful information on the three-dimensional structure of the Thr-GalNAc part, which can be applicable to other mucins. Essential results from Q-mucin obtained by several NMR techniques used here will be a guide for analyses of more complex mucins in natural abundance.

Results and Discussion

Because the monosaccharide composition fluctuates in sample after sample, we selected one sample from *Aurelia aurita* with the simplest composition and with minimum content of the unidentified monosaccharide. The main four saccharides were GalNAc, Gal, Ara (arabinose), and unidentified X. Similarly to the previous study,[21] no sialic acids were found in the sugar composition. We expected from the composition summarized in Table 1 that the majority of the NMR signals belong to GalNAc or X and that those from the two minor components, Ara and Gal, tend to fade into the background.

The chemical shifts of protons and ^{13}C's in the peptide chain were estimated by a combination of several techniques including DQF-COSY, HMQC/HSQC, HMBC, DPFGSE (double pulsed field gradient spin echo)-TOCSY, and DPFGSE-NOE.[23−26] In glycoproteins, the signals from anomeric protons of saccharides and α-protons appear in a similar area of chemical shifts. The correlation analyses with ^{13}C's for each of the protons were helpful to confirm the assignments. However, full analysis of all cross-peaks appearing in these 2D spectra was impossible owing to the complexity of the spectra, the inhomogeneous property of mucin as a polymer sample extracted from natural sources, and serious overlaps of peaks. For the present sample, any isotope labeling or concentration techniques would be difficult to use for magnifying the appropriate signals to help in the full analysis. Therefore, to avoid a very long period of accumulation and calculation, we chose significant peaks and performed several 1D-type measurements to clarify two essential structures, i.e., the amino acid sequence and connectivity around the *O*-glycoside bonds. The main content of this paper focuses only on the results obtained using this strategy.

The assignments of all the protons and ^{13}C's in the peptide chain were satisfactorily obtained in reference to the results of the model peptide (MP), a peptide of eight amino acids identical to the single tandem repeat of Q-mucin but with no saccharide chains connected (R = H and $n = 1$ in Scheme 1). A comparison of the chemical shift between Q-mucin and MP is summarized in Table S1 in the Supporting Information (SI). Although no large disagreements with classical studies[13−19] on OSM were found, the accuracy of this study is improved because new pulse techniques were applied. The signals from duplicated residues at different positions (Ala, Thr, and Val) were well separated as numbered from the N-terminus, such as Val(1), Val(2) as shown in Scheme 1. The consistency of all these assignments was also confirmed satisfactorily in all 2D spectra.

We also visually summarize the difference in chemical shifts between Q-mucin and MP in Figure 1. The bar graph indicates the difference between the observed values for the ^{13}C or proton at identical positions. While the changes occurring in the two terminal residues (Val(1) and Pro) are relatively large, those of the two Thr's are noteworthy in terms of *O*-glycosylation. Because no unglycosylated Thr was identified in the present observation, most of the existing Thr appears to be covered with glycochains forming *O*-glycoside bonds.

Figure 2 shows the result of the 2D ^{1}H-selective HMBC measurement in the cross-peak area between the signals from the α-protons and the ^{13}C signals in the bridging carbonyl groups of the peptide bonds as shown in Scheme 1. The sequence of −VVETTAAP− can be traced on the cross-peaks.[27] In accordance with the previous result, the existence of the minor −VIETTAAP− sequence was also implied in Figure 2. Although the same attempt was made for MP, several cross-peaks were clear in D_2O and none were obtained in DMSO. This is probably because of the extensive vibrations of the small peptide unit without saccharide branches.

The appearance of cross-peaks in Q-mucin also suggests the existence of partial restriction in the configuration of the peptide chain. This restriction is not as tight as that observed in the α-helix or *β*-sheet. Generally, total NMR spectra obtained from mucins in solutions including Q-mucin and OSM showed well-resolved line shape with motional narrowing. As supported by theoretical calculations[20] for the model peptide of 15 residues for MUC1, we consider that the entire polymer is a flexible cord probably forming a linear long chain similar to polysaccharides. Ribbonlike shapes of mucins visualized by atomic force microscopy (AFM) also suggest a stretched structure of the peptide main chain.[28,29] However, the local structure within the tandem repeat unit is moderately restricted owing to the crowded structure with the presence of saccharide chains.

In the area depicted in Figure 2, no other strong cross-peaks except those involved in the tandem repeat were observed. This fact and the previous result[21] obtained from the analysis of amino acid components imply that the Q-mucin extracted by the present

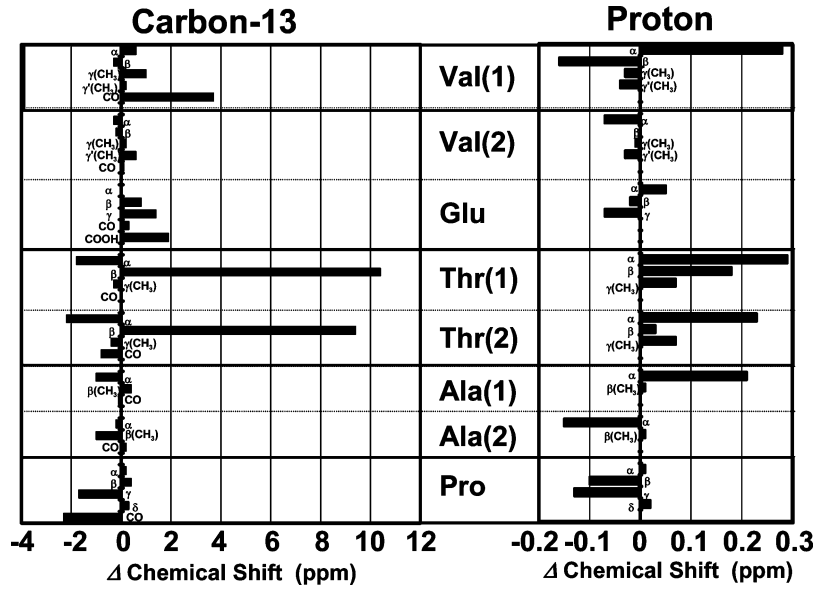


Figure 1. Bar graph indicating the difference in chemical shifts of various carbon-13 and protons in amino acid residues between model peptide (MP) and qniumucin (Q-mucin) ($\Delta = \delta_{\text{Q-mucin}} - \delta_{\text{MP}}$). Values in residues at both ends (Val(1) and Pro) and central Thr(1) and Thr(2) show large differences.

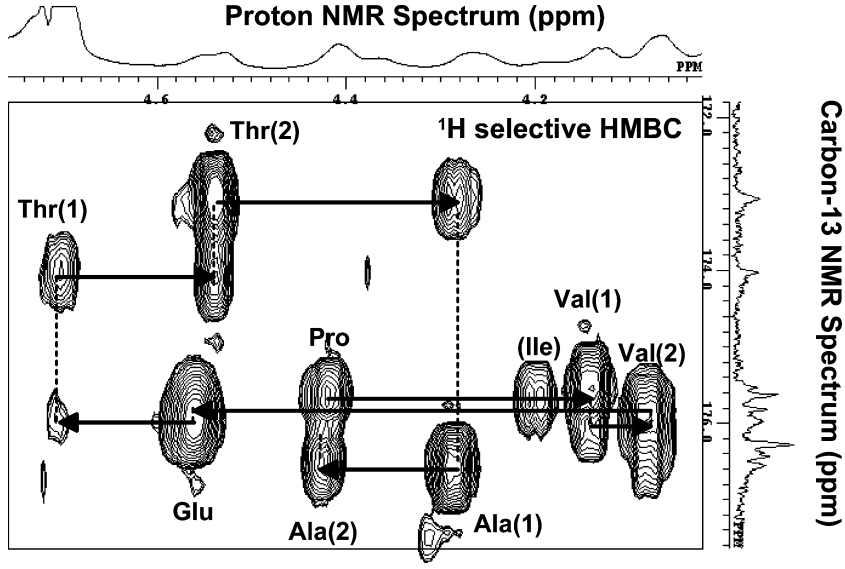


Figure 2. 2D ^{1}H selective HMBC spectrum of Q-mucin showing the interactions between α-protons and carbonyl ^{13}C's on the peptide bonds. The amino acid sequence −VVETTAAP− in the tandem repeat can be traced by connecting each correlation peak. A small amount of −VIETTAAP− sequence is also revealed.

protocol is composed of only the sequential tandem repeat part despite a wide distribution of molecular mass as previously shown.[21] The results of Edman degradation in our previous paper[21] showed that the N-terminus started from the sequence of the tandem repeat. Moreover, we attempted to stain Q-mucin using general protocols available for proteins, i.e., Coomassie brilliant blue (CBB) and silver staining methods, which were unsuccessful, indicating the lack of sensitive moieties for these methods in the total sequence. All these results support our speculation that Q-mucin is composed of a monotonic sequence of tandem repeat units. Different from the majority of mucins discovered to date, Q-mucin has an exceptionally high homogeneity, which is an advantage if it is to be used as an industrial material.

The simple structure of Q-mucin shows a sharp contrast to those of higher metazoa such as mammals. The apparent difference is the lack of extra domains such as von Willebrand D (VWD) and cysteine-knot domains, which are responsible for the oligomerization of mucin molecules.[30] Recent bioinformatic analysis suggests that gel-forming mucins appeared early in metazoan evolution based on the presence of their genes in a starlet sea anemone, which belongs to the same division, Cnidaria, as jellyfish.[31] Therefore, without any information about the genome of the present jellyfish, we speculate that Q-mucin also belongs to the gel-forming mucins as well.

For the NMR assignments of protons and ^{13}C's involved in the saccharides, only those of GalNAc were clearly identified as shown in Table S2. Although two types of GalNAc were recognized, they are found to connect with different threonines, Thr(1) and Thr(2) (*vide infra*). The question is why we cannot identify any signals from X separately. At this moment, we consider two possibilities: (1) X gives an NMR spectrum similar to that of GalNAc and (2) a large difference in relaxation time exists between GalNAc and X. We will discuss this at the end of the section.

Next, the connectivity between Thr and GalNAc in the glycobranches was confirmed using various NMR techniques. A conventional HMBC measurement showed small cross-peaks for the

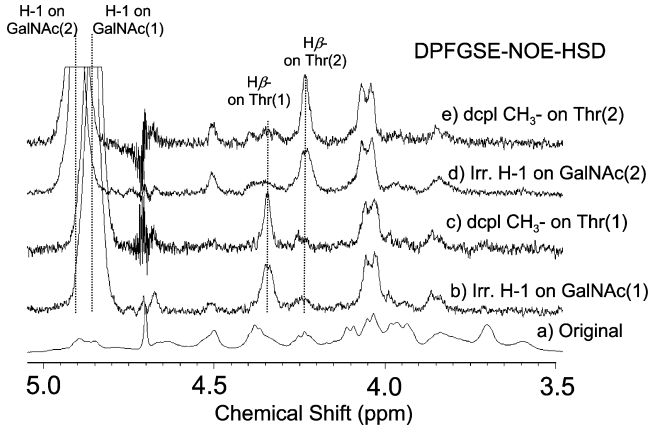


Figure 3. Results of DPFGSE-NOE-HSD measurement used as a key analysis of the connectivity between Thr and GalNAc. Either by the decoupling of methyl protons on Thr (c, e) or the irradiation of H-1 on GalNAc (b, d), the peak of the nearest β-protons was sharpened. The original proton NMR spectrum is shown for reference as line "a)".

β-carbon of Thr versus H-1 in GalNAc and those for the β-proton of Thr versus C1 in GalNAc, indicating that both Thr residues are independently coupled with a single GalNAc. All the NOEs observed in the DPFGSE-NOE spectrum (2D NOESY spectrum is shown in Figure S3 in the SI) showed negative values. An NOE was observed from the H-1 signal of GalNAc(1) to the H signal at 4.38 ppm, which is assigned to the β-proton of Thr(1) on the basis of the results of the TOCSY experiment. Similarly, an NOE between the H-1 signal of GalNAc(2) and the β-proton of Thr(2) was also detected. Each Thr has a methyl group, the proton of which showed NOEs with its own α-proton, its β-proton, and H-1 of GalNAc. Of note is the existence of the methyl group in Thr (in contrast to Ser), which greatly helped us to trace the NOE through the *O*-glycoside bonds because the relaxation times of their protons are appropriately short and close to that of the H-1 of GalNAc.

In the conventional 2D HMBC spectrum, however, several days were necessary to obtain only a minimally informative result, in which the two Thr−GalNAc pairs were not separated. To further confirm the Thr−GalNAc connectivity, a type of 1D measurement, DPFGSE-NOE-HSD,[32] was performed, as shown in Figure 3. Under the experimental condition where NOE was observed between H-1 and the β-proton, the protons in each methyl group were independently decoupled, resulting in narrowing of a single peak of the adjacent β-proton. Consistently, the spin coupling constants between α- and β-protons have been reported to be less than 2 Hz in the literature.[33] Because all the information was obtained in several hours with this strategy, this approach is generally convenient for analyzing unknown samples of *O*-glycans including mucins. The results of connectivity analyses are summarized in Figure 4 with a molecular structure. This is the first confirmation for Q-mucin that the connecting carbon of GalNAc to Thr is C1.

TOCSY measurements in 80% H_2O/20% D_2O solution also provided information on the configuration of the Thr−GalNAc local group. The CH_3− of Thr was irradiated to observe the *J*-coupling of the β-protons with the surrounding protons. In MP, the energy flow swiftly reached the α-proton and the nearest NH-proton within a short time. However, this interaction was significantly delayed in Q-mucin, as shown in Figure S5 in the SI. Apparently, there is a gap of energy transfer between α- and β-carbons of threonines in Q-mucin, which is caused by the addition of the glycan chain on the peptide. On the basis of a primitive molecular mechanics (MM) calculation with an MM2 program that suggests a weak repulsion between the peptide chain and glycochain, the Newman projection figure shown on the right side of Figure 4 indicates that GalNAc and H_α tend to take a near-gauche position with a hindrance of bulky groups. As a result, $H_\beta-C_2-C_1-H_\alpha$ appears to be restricted around the near-vertical position to minimize *J*-coupling between H_α and H_β. As shown in Figure 4, these two protons seem to be restricted at a close position to magnify their NOE. This speculation is also supported by the detection of a small NOE interaction from the anomeric proton of GalNAc to the α-proton of the nearest Thr. Recognition of this small NOE was impossible in 2D NOESY observation in Figure S2 but possible in a 1D experiment with selective irradiation.

The present NMR study indicates that the local structure of mucin around the *O*-glycoside bond (GalNAc−Thr) is relatively inflexible owing to the repulsion between the peptide main chain and the saccharide chain. Although the present NMR study provides information on the local structure of only one type (GalNAc−Thr), this can be regarded as a general situation for any type of mucin because we can extend this consideration to the other types of *O*-glycoside bond (GalNAc−Ser) where an effective NMR measurement is difficult for polymers without $-CH_3$ at the β-position. The small motif of the GalNAc−Ser group without the peptide chain was investigated by NMR and a theoretical approach by Avenoza et al.[19] Their conclusion is that the restriction of the orientation around the *O*-glycoside bond is very weak and that the effect of surrounding water molecules must be considered such as ones tightly bind to the local structure via hydrogen bonds. A similar effect of solvation can be considered in our present system.

Moreover, in Q-mucin, two bulky GalNAc−Thr groups are adjacent to each other in the sequence of the tandem repeat, and a strong interaction (or repulsion) between the two saccharide side chains causes another restriction of the orientation of the peptide chain. This partly explains the contrast in our present HMBC results between Q-mucin (in Figure 2) and MP, implying the difference in the orientation and the dynamics of the peptide main chain. Considering that the existence of the *O*-glycoside bond (GalNAc−Thr or GalNAc−Ser) is one of the top priorities in the definition of mucins, it is noteworthy that its local structure, which is inflexible and bulky, has significant effects on the main chain structure and its dynamics in larger scale, particularly for the peptide main chain. A ribbonlike shape in the AFM observation of mucins may be one of the most apparent results.[28,29]

Other than those from GalNAc, no clear proton or ^{13}C signals from the saccharides were identified, probably because of the existence of glycoforms that hide the other components in the background. Most of the glycochains of the present HPLC fraction seem mainly composed of GalNAc, including substituted ones consistent with the components indicated in Table 1. No separate signals that can be attributed to X in the monosaccharide composition were obtained. On DEPT measurement, we did observe two separate ^{13}C signals of C-6 in GalNAc at 63.8 and 65.9 ppm, the latter of which showed a low-field shift of approximately 2 ppm, which implies the substitution by some NMR-silent group or minor groups such as other saccharide chains or other moieties. Because substitution with another saccharide provides a low-field shift of more than 8 ppm,[36] we considered the substitution by functional groups such as $-SO_3^-$ or $-PO_3^{2-}$. We performed ^{13}C NMR measurement on two standard materials, *N*-acetyl-D-galactosamine-6-*O*-sulfate and *o*-nitrophenyl-1β-D-galactopyranoside-6-*O*-phosphate, which are commercially available, and obtained the chemical shifts of C-6 of 70.0 ppm (∼6 ppm shift) and 65.5 ppm (∼2 ppm shift), respectively. The result shows that $-PO_3^{2-}$ is more probable than $-SO_3^-$. However, these esters should be removed on hydrolysis before the monosaccharide analysis and cannot be detected as different components. We also speculated that the unidentified saccharide (X) may be a substituted GalNAc because X has a long retention time that is comparable to that of GalNAc in HPLC[34,35] (see Experimental Section), implying that it may be a polar monosaccharide similar to GalNAc. Therefore, another derivative

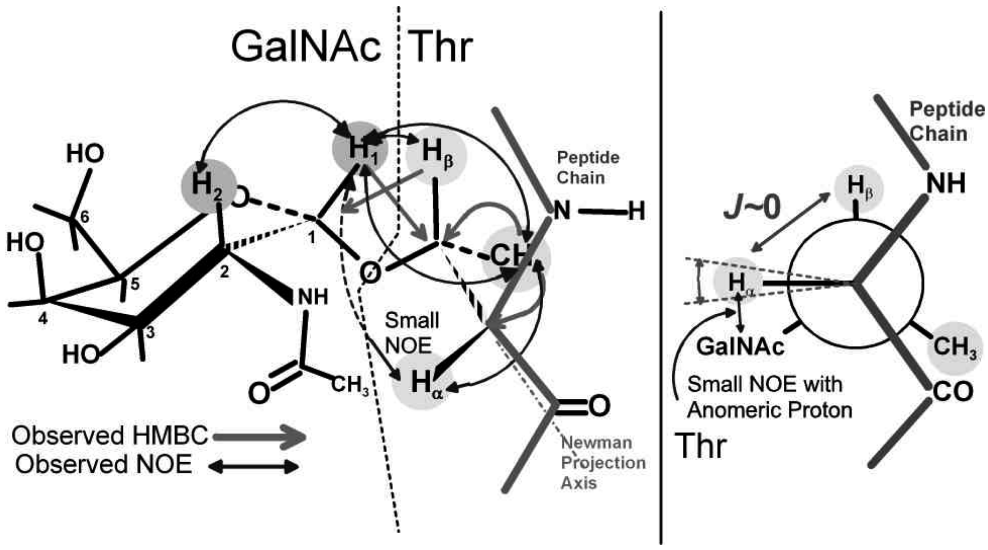


Figure 4. Summary of the connectivity of the GalNAc−Thr unit in qniumucin, which was assigned on the basis of NMR measurements, including indications of HMBC, NOE, and *J*-coupling. Left: Roughly drawn stereostructure of GalNAc−threonine unit with connecting arrows indicating HMBC (single-sided arrows with a thick line) and NOE (double-sided arrows with a thin line) interactions. A small NOE is also observed between the anomeric proton (H-1) of GalNAc and the α-proton (H_α) of Thr. Right: Probable configuration around $H_\beta-C_2-C_1-H_\alpha$ is indicated in a Newman projection. *J*-Coupling between H_α and H_β is almost zero, while the NOE from the anomeric proton (H-1) of GalNAc to H_α of Thr is small. Therefore, H_α is likely to be restricted in a gauche or near-gauche position in reference to GalNAc and a vertical or near-vertical configuration in reference to H_β.

of GalNAc, which is resistant to hydrolysis using normal protocols, is a candidate for X, which is still unknown in this study.[37]

In conclusion, the present NMR study on Q-mucin provided an appropriate strategy to solve the structure of mucins with natural isotope abundance. Because of the significant overlap of chemical shifts of ^{13}C and proton signals in peptides and saccharides, typically the anomeric protons and α-protons, the interfacial connectivity through the *O*-glycoside bond is difficult to clarify in a polymer of high molecular mass. However, our results of Q-mucin, which has moderate relaxation times owing to the extensive motion of the peptide main chain, showed that the irradiation of CH_3- in Thr was effective for TOCSY, NOESY, and HMBC observations. Consequently, a detailed structural analysis was achieved as shown in Figure 4 for the core structure of mucin, Thr−GalNAc, which is generally found in other mucins. It was found that several 1D DPFGSE techniques (DPFGSE-TOCSY, DPFGSE-NOESY, and DPFGSE-NOE-HSD) were useful in combination with popular 2D techniques (FG-HMBC, DQF-COSY, FG-HSQC/HMQC) for studying the complex polymeric samples. The high homogeneity of Q-mucin is also proved, indicating its superiority over other inexpensive mucins, such as the gastric mucins from domestic animals.

Experimental Section

Qniumucin (Q-mucin) was extracted from *Aurelia aurita* caught in Hakata Bay, Fukuoka, Japan, by the method reported in our previous paper.[21] Further purification was performed by ion-exchange HPLC. A TSKgel DEAE-5PW column (7.5 mm i.d. × 75 mm) from Tosoh Corp. was used with a gradient of 0 → 100% of B (0.5 M NaCl/10 mM sodium phosphate buffer, pH 7) into A (10 mM sodium phosphate buffer) in 0−20 min at a flow rate of 0.5 mL/min. We monitored two wavelengths for sugar (215 nm) and proteins (275 nm). The main peak fraction monitored at 215 nm was isolated as a solid after freeze-drying.

Two standard reagents for ^{13}C NMR measurement, *N*-acetyl-D-galactosamine-6-*O*-sulfate (sodium salt, mixed anomers) and *o*-nitrophenyl-1β-D-galactopyranoside-6-*O*-phosphate (cyclohexyl ammonium salt), were obtained from Dexta Laboratories, UK, and Toronto Research Chemicals Inc., Canada, respectively, and used as received.

The chromatographic analysis of sugar components was performed after hydrolysis using 4 M trifluoroacetic acid for 3 h at 100 °C. The sample was *N*-acetylated and labeled using a fluorescent label (ABEE) and then analyzed using a Honenpak C18 column (4.6 mm i.d. × 75 mm) from J-Oil Mills Inc., 0.2 M potassium borate buffer (pH 8.9) containing 7% CH_3CN, and a standard kit from J-Oil Mills Inc. containing 11 monosaccharides.[34,35] The obtained monosaccharide composition of this particular sample is shown in Table 1.

Approximately 25 mg of purified Q-mucin was dissolved in 0.12 mL of D_2O and sealed in a 5 mm NMR microtube from Shigemi. While the bulk sample easily dissolved in D_2O, a small amount (<5%) of insoluble residue was identified even after 7 days and was finally removed by decantation and filtration. Therefore, further concentration of the water (D_2O)-soluble component was achieved in the final step. Some 2D data (FG-COSY, FG-NOESY, FG-HMBC, and FG-HSQC) and DPFGSE-TOCSY were obtained from the sample dissolved in 80% H_2O/20% D_2O.

The model peptide (MP) was synthesized using a ABI433A peptide synthesizer (Applied Biosystems Inc.) and purified by HPLC at the Support Unit for Biomaterial Analysis, Research Resource Center, Brain Science Institute (BSI), Riken, Wako, Saitama. The MP obtained was used without further purification.

The chemical shifts reported in this paper are those in ppm from sodium 2,2-dimethyl-l,2-silapentane-5-sulfonate (DSS) used as an external standard. All spectra were measured at 30 °C. The 2D spectra, DPFGSE-NOE, and TOCSY spectra were recorded on a JEOL ECA-600 spectrometer (both at Riken and Chiba University), with a magnetic field of 14 T, equipped with an HCX5FG2 and TH5ATFG probe head and an actively shielded Z-gradient coil. The HMBC and ^1H-selective HMBC spectra were both optimized for 8 Hz. Their data points were 2K in the F2 frequency domain and 512 increments of evolution time, t1. Both experiments took about 4 days. The proton-selective Gaussian pulse width used for the latter was 4 ms, and the selective proton chemical shift was 4.43 ppm. The DPFGSE NOE with homospin-decoupling (HSD) spectra was recorded on a JEOL alpha-400 spectrometer of Riken with a magnetic field of 9.4 T, equipped with a TH5FG probe head and an actively shielded Z-gradient coil. The length of the proton-selective Gaussian pulse was 66 ms. The power level for HSD was

the same as that of the original 1D HSD condition (8 kHz time-sharing decoupling). Mixing time in NOE was 400 ms. Repetition rates were 4−5 s (acquisition time + pulse delay). The period needed to complete the total experiment was 50 min under each condition.

MM2 calculations were performed with a ChemOffice program on a personal computer.

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Supporting Information Available: Table of assigned chemical shifts, full 2D-NMR DQF-COSY, FG-NOESY, FG-HMBC, and FG-HSQC spectra, and the experimental results of DPFGSE-TOCSY are provided. This material is available free of charge via the Internet at http://pubs.acs.org.

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(37) Recently, during revision of this paper, the authors and a co-worker achieved the determination of species X using ^{31}P NMR and high-resolution mass spectrometry on a fractionated sample from monosaccharide analysis in Table 1. The result will appear in a separate publication. Urai, M; Nakamura, T.; Uzawa, J.; Baba, T.; Taniguchi, K.; Seki, H.; Ushida, K. Manuscript in preparation.

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