Ligand-Aided $^1$H Nuclear Magnetic Resonance Spectroscopy for Non-Destructive Estimation of Sulfate Content in Sulfated Saccharides

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Abstract

Sulfated saccharides exhibit diverse physiological activities, but a lack of any convenient assay hinders their evaluation. Herein, an assay for the analysis of sulfated saccharides is developed using $^1$H nuclear magnetic resonance (NMR) spectroscopy by employing ligands that can form ionic complexes with the sulfate groups. Based on the change in the chemical shift ($\Delta \delta$) of the ligands by sulfated mono- to tetrasaccharide, imidazole was found to be a good ligand showing the maximum $\Delta \delta$; neutral saccharides do not show any change in the $\delta$ value. A marked and constant downfield $\delta$ value observed was changed dramatically at molar ratio of > 1:1 (imidazole:sulfated saccharides), allowing the sulfate content estimation based on the concentration of imidazole at the $\Delta \delta$ inflection point. By the proposed ligand-aided $^1$H NMR assay, the sulfate content of natural sulfated polysaccharide, fucoidan, was non-destructively estimated to be 0.21 mmol/g-fucoidan.

Keywords sulfated saccharide, imidazole, $^1$H NMR, ligand, fucoidan, non-destructive assay
Introduction

Sulfated polysaccharides are commonly found in viscous components covering the cell walls of seaweeds, where the sulfate groups are covalently bonded to the sugar backbone. Marine sulfated polysaccharides including agarans, carrageenan, and fucoidan, have been reported to exhibit various physiological functions such as anti-coagulant, anti-angiogenic, anti-cancer, and immunostimulatory activities.1-6 Interestingly, such biological activities are closely associated with the sulfate content of saccharides.1,2,5,7 You et al.6 reported that the sulfate group in fucoidan bonded to the cationic proteins on human gastric carcinoma cells, exhibiting sulfate content-dependent anti-proliferation activity. Haroun-Bouhedja et al.1 reported that Ascophyllum fucans containing > 20% of sulfate content exhibited potent anti-coagulant and anti-proliferative activities. Moreover, Koyanagi et al.2 revealed that synthesized fucoidan containing > 50% of sulfate content inhibited the binding of vascular endothelial growth factor 165 to the receptors expressed on vascular endothelial cells, showing anti-proliferation and anti-migration of the cells.

Despite the significant physiological benefits of sulfated saccharides, the understanding of the relationship between the sulfate groups and various physiological effects remains unclear. A common assay for determining the sulfate content of sulfated saccharides is performed using acid hydrolysis, followed by the precipitation of inorganic sulfate with barium chloride.8 This method requires tedious extraction and purification procedures prior to ultraviolet (UV) spectroscopic determination.2,6 In addition, this assay can suffer from the low reproducibility of extraction and tedious experimental procedures. Therefore, a convenient and reliable assay for the estimation of sulfate content of bioactive saccharides is required.

Owing to the non-destructive analysis afforded by nuclear magnetic resonance (NMR) spectroscopy, recent studies have focused on the application of NMR in quantitative assays that allow simple, reliable, and non-destructive estimation of food quality, for example, glucose assay for fruit juices,9 sucrose assay for beverages,10 and formic acid assay for apple juices.11 However, to realize the NMR-aided
assay for the analysis of sulfated saccharides, the target protons in saccharides must be well resolved from other proton signals. Although the commonly targeted anomeric protons in saccharides are observed at chemical shifts (δ) of 4.5–5.8 ppm in the 1H NMR data, interferences from the matrix typically overlap with the signals of the anomeric protons. For sulfated saccharides, the δ values of the anomeric protons at 4.5–5.8 ppm were shifted to higher magnetic fields owing to the substituent effect, resulting in an overlap with the δ values of other protons from the saccharide skeleton. In this study, an NMR titration method is applied for determining the sulfate content in sulfated saccharides by monitoring the δ of the ligand that can form a complex with the sulfated saccharide. The NMR titration method is used to design artificial receptors for anions by monitoring the changes in the chemical shifts (Δδ) of the receptor protons. A thiourea-based tripodal receptor (3-nitrophenyl-based tripodal tris-thiourea) showed a marked Δδ when an equivalent mole of sulfate anion was complexed to the receptor. A significant Δδ caused by the 1:1 complex formation of the target with the ligand has been reported in prior literatures. In the present study, a suitable ligand molecule to form complexes with the sulfated saccharides was selected by screening various ligands, and the application of the proposed ligand-aided 1H NMR assay for determining the sulfate content was evaluated. Sulfated saccharides used in this study include the commercially available mono- to tetrasaccharides with definite sulfate contents (Fig. 1). Sulfate content in commercially available natural sulfated polysaccharide, fucoidan, was also evaluated by the proposed ligand-aided 1H NMR.

**Experimental**

**Reagents**

D-(+)-Galactose (Gal), L-arginine, glycine, barium chloride dihydrate, L-ascorbic acid, sodium sulfate, and imidazole were obtained from Nacalai Tesque Inc. (Kyoto, Japan). Galactose-6-O-sulfate (G6S), neocarrabiose-4-O-sulfate (NB4S), neocarratetraose-41,3-di-O-sulfate (NTdS), 3β-galactobiose (β-
Gal-[1→3]-Gal) (GalB), and 3α-4β-3α-galactotetraose (α-Gal-[1→3]-β-Gal-[1→4]-α-Gal-[1→3]-Gal) (GalT) were purchased from Dextra Laboratories Ltd. (Shinfield, UK); all sulfated chemicals were in the form of sodium salts. D- (+)-Galacturonic acid monohydrate, piperazine hexahydrate, and 3-(trimethylsilyl) propionic-2,2,3,3-d4 acid (TSP-d4) were purchased from Wako Pure Chemical Co. (Osaka, Japan). Deuterium oxide (D2O, 99.8 atom% D), 1-(2-pyrimidinyl) piperazine, and sodium rhodizonate were obtained from Kanto Chemical Co. Inc. (Tokyo, Japan). Fucoidan from Fucus vesiculosus was purchased from Sigma-Aldrich Co. (≥ 95% pure, Cat. No. F8190, St. Louis, MO, USA).

1H NMR measurement

One-dimensional (1D) 1H NMR measurements were collected at 25°C using an ECS-400 spectrometer (JEOL, Tokyo, Japan) to determine the $\Delta\delta$ value of the ligand in D2O containing TSP-d4 as an external standard. A single pulse sequence was used for acquiring the NMR spectra employing an acquisition time of 2.18 s, 16,384 acquisition data points, 8 scans, relaxation delay of 15 s, and spinning at 15 Hz. The 1H NMR spectra were referenced using TSP-d4 at 0.00 ppm. To prevent any unexpected interaction of the ligand with TSP-d4 bearing a carboxyl group, TSP-d4 dissolved in D2O was packed into a 3.5-mm-stem coaxial insert NMR sample tube (Nihon Seimitsu Scientific Co., Tokyo, Japan), and the insert tube was repeatedly used for all samples by inserting it into a 5-mm NMR sample tube containing 400 µL of the sample solution (Nihon Seimitsu Scientific Co.).

Preparation of ligand-sulfated saccharide solution

To prevent the effect of Na+ on the binding of ligand to sulfate group in saccharide, prior to the preparation of the ligand-sulfated saccharide solution, sulfated saccharide dissolved in D2O was passed through a TOYO-PAK IC-SP M cartridge (TOSOH Co., Tokyo, Japan) to eliminate Na+ from the D2O solution. The ligand dissolved in D2O was then mixed with the saccharide solution in 0.2:1 to 3:1 molar
ratios of the ligand to the saccharide. The molar concentration of the saccharide in the solution was 8.3 mM for all tested samples. Gal, GalB, and GalT bearing no sulfate groups were used as negative controls. The esterification of carboxylated monosaccharide was performed to determine the effectiveness of the esterification treatment to eliminate the unexpected affinity of the ligand to not only the sulfate groups, but also carboxyl groups in the saccharides. Galacturonic acid, a carboxylated monosaccharide, was esterified in this experiment, following the method reported by Jansen and Jang\textsuperscript{19} with some modifications. Briefly, galacturonic acid was dissolved in 25 mM HCl/methanol (MeOH) solution and incubated for three days at 4°C, followed by neutralization with 1 M KOH/MeOH solution. After neutralization, the solution was evaporated under N\textsubscript{2} atmosphere. The residues were dissolved in distilled water and passed through TOYO-PAK DEAE and TOYO-PAK IC-SP M cartridges (TOSOH Co.) to remove the remaining galacturonic acid and potassium ion, respectively. The filtrate was lyophilized, and the dried methyl-\textalpha-D-galacturonate (or methyl galacturonate) dissolved in D\textsubscript{2}O was used for subsequent \textsuperscript{1}H NMR measurements.

Assays for sulfate content of F. vesiculosus fucoidan

A conventional assay for sulfate content in fucoidan was performed by a barium chloride-rhodizonate method after the hydrolysis of fucoidan (10 mg) with 2 mL of 3 M HCl at 100°C for 2 h.\textsuperscript{20} Briefly, an aliquot (0.5 mL) of the hydrolyzed fucoidan was mixed with 1.0 mL of 10 mM barium buffer, 1.5 mL of 50 µg/mL rhodizonate, and 2.0 mL of ethanol, and incubated for 10 min at room temperature. After the incubation, absorbance of the solution was measured at 520 nm. The standard curve (sodium sulfate as standard) covered the range of 0–24 µg/mL of sulfate. To evaluate the sulfate content of fucoidan by the ligand-aided \textsuperscript{1}H NMR, ligand-fucoidan solution was prepared according to the method as described above. Imidazole at difference concentrations of 1.25–6.25 mmol-ligand/g-fucoidan was mixed with fucoidan dissolved in D\textsubscript{2}O (0.08 mg-fucoidan/mL).
**Statistical analysis**

Sulfate content in fucoidan by the conventional method was measured in triplicate and presented as the mean ± standard deviation (SD). Unpaired Student's *t*-test was used to compare the difference between sulfate contents obtained by conventional and NMR methods.

**Results and Discussion**

**Screening of ligands for \(^1\)H NMR-aided sulfate content assay of sulfated saccharides**

In this study, candidates responsible for preferable complex formation with target sulfated saccharides are required having counter-ionic properties. Some researchers have reported that azacyclic compounds\(^{13,14,17}\) and ammonium/guanidinium group\(^{15,16,18}\) can selectively interact with sulfate ion, allowing us to use imidazole, piperazine, L-(2-pyrimidinyl) piperazine, glycine, and L-arginine, as typical azacyclic and ammonium compounds in the present ligand-aided \(^1\)H NMR assay (Fig. 1). Fig. 2 shows the δ values of the ligands in the presence or absence of a monosulfated disaccharide, NB4S, with 1:1 molar ratio (ligand:saccharide). Among the five candidates, marked downfield shifts in the δ values (Δδ > 0.15 ppm) were observed for imidazole, piperazine, and glycine, whereas no or relatively small shifts were found for L-arginine and L-(2-pyrimidinyl) piperazine. For L-arginine, the characteristic protons were not clearly observed because of signal broadening after the mixing of NB4S. Although significantly high Δδ values of 0.28 and 0.15 ppm were observed for piperazine and glycine, respectively, by the formation of NB4S complex, their δ values were at approximately 3.5 ppm, which are likely overlapped with those of the saccharide protons.\(^{12}\) The overlapped proton regions observed for ligands and saccharides also exclude further investigation on other possible ligands, e.g., lysine in this study. In contrast, imidazole showed distinct shifts of the δ values of the aromatic protons to a low magnetic field in the presence of NB4S (H4,5: 7.15 to 7.45
ppm; H2: 7.80 to 8.59 ppm). This observation is consistent with that report by Ihm et al.,\textsuperscript{13} who described a large downfield shift of the δ value of imidazolium H2 protons in tripodal nitro-imidazolium receptor upon the addition of chloride and bromide anions. However, because of the lower intensity of H2 signal (Fig. 2) in comparison to that of the H4,5 signal with δ at a lower magnetic field (7.45 ppm), which is distinguishable from δ values of the saccharide protons, the H4,5 signal was selected as an appropriate indicator of the imidazole ligand activity for sulfated saccharides in further experiments.

**Sulfate content of neocarrabiose-4-O-sulfate by ¹H NMR**

To determine the effect of the complex formation of imidazole-sulfated saccharides on the δ values of the imidazole protons (Fig. 2), the δ values of the imidazole protons (H4,5: 7.15 ppm, only imidazole in D\textsubscript{2}O) were investigated as a function of the molar ratio of imidazole to NB4S (0.2:1 to 3:1). As shown in Fig. 3A, no changes in the δ values of the imidazole protons were observed up to a molar ratio of 1:1, while at higher molar ratios of >1:1, the δ value shifts dramatically toward higher magnetic fields likely due to the free or over-saturated imidazole molecules. No change in the δ value up to a saturated molar ratio (1:1) indicates that an imidazole molecule can ionically interact and form a complex with the sulfate group of the monosulfated disaccharide, NB4S. This is consistent with no change reported in the δ value of the imidazolium protons in tripodal nitro-imidazolium receptor at < 1:1 molar ratio of the receptor to chloride and bromide anions in prior literature.\textsuperscript{13} The effect of solution viscosity on δ value can be excluded, as ¹H NMR signal broadening was not observed with an increase in solution viscosity\textsuperscript{21} under the experimental conditions (fixed concentration of 8.3 mM of saccharides) in this study (Figs. 3A, 4, and 5). As shown in Fig. 3B, plotting of the observed δ as a function of molar ratio (imidazole vs. NB4S) can allow the sulfate content estimation in sulfated saccharides based on the inflection point of the plot. When δ is plotted as a function of the cumulative molar concentration of imidazole, the concentration of imidazole at the inflection point corresponds to the sulfate content in saccharides.
Complex formation of imidazole with sulfated saccharides by $^1$H NMR

Understanding of the stoichiometric complex formation of an imidazole molecule with the sulfate group of the saccharide remains elusive, as the complex formation of the ligands with the saccharide sulfate groups is influenced by the number of sulfate groups and/or molecular size and structure of the sulfated polysaccharides.6,22 Thus, to confirm the stoichiometric complex formation of an imidazole molecule with the saccharide sulfate group in this study, the $\Delta\delta$ value of imidazole was examined using monosulfated mono- and disaccharides (G6S and NB4S, respectively) and disulfated tetrasaccharide, NTdS. Their corresponding non-sulfated saccharides, namely, mono-, di-, and tetrascarharides, i.e., Gal, GalB, and GalT, respectively were used as negative controls. The molar ratio of all the tested mixtures was set at 1:1; for disulfated NTdS 16.7 mM imidazole was mixed with 8.3 mM NTdS (imidazole:sulfate group of NTdS = 1:1). As shown in Fig. 4, no change in $\delta$ of the imidazole protons (7.15 ppm) was observed upon the addition of non-sulfated saccharides, whereas a marked $\delta$ change in the $^1$H NMR data was observed upon adding sulfated saccharides. The same magnitude of $\Delta\delta$ in the presence of each sulfated saccharide together with its corresponding non-sulfated saccharide clearly indicates that an imidazole molecule specifically recognizes the target sulfate group in the sulfated saccharides (mono- to tetrascarharides), regardless of the contaminating saccharides. Smaller $\Delta\delta$ values of the imidazole protons (H4,5) of G6S ($\Delta\delta$: 0.23 ppm) and of NTdS ($\Delta\delta$: 0.22 ppm) compared to that of NB4S ($\Delta\delta$: 0.29 ppm) can be possibly due to the lower affinity of imidazole to the sulfate groups attached to different position in G6S and NTdS. Factors affecting the magnitude of $\Delta\delta$ in sulfated saccharides must be investigated using diverse saccharides with different size and binding position of sulfate group in future. Although association constant between imidazole and sulfate group in saccharides by an NMR titration analysis13,15 is required for investigating the factors, the reported association constant of imidazole with sulfate ion in DMSO-$d_6$ (350 M$^{-1}$)13 that is in the range of sufficient binding ability of ligand to anions (80 M$^{-1}$).
for Br⁻ to > 10⁴ M⁻¹ for SO₄²⁻)¹⁵ suggested that the imidazole in this study may play as a moderate ligand binding to sulfated saccharides in D₂O solution.

Notably, in addition to the non-sulfated and sulfated groups, the carboxylated groups such as galacturonic and alginic acids³,²³ are present in natural marine saccharides. Ihm et al.¹³ reported that bis-imidazolium formed a complex with carbonate as well as sulfate. Therefore, saccharides with carboxyl groups can affect the formation of imidazole-sulfate complexes or the estimation of sulfate content in sulfated saccharides by the ligand-aided ¹H NMR assay described in this study. For extensive application of this assay to determine the sulfate content in natural saccharides, the elimination of imidazole-coupling carboxyl groups is, thus, needed prior to ¹H NMR measurements. As shown in Fig. 5, galacturonic acid, a typical marine carboxylated monosaccharide, showed a downfield shift of the δ value of the imidazole protons (7.15 ppm to 7.47 ppm), which was similar to that observed for the sulfated saccharides (Fig. 4). Considering its pKa (3.24),²⁴ galacturonic acid dissolved in D₂O can potentially allow the formation of a complex between imidazole and ionized galacturonic acid in a manner similar to that observed for the sulfated saccharides (Fig. 4; sulfate group as a strong acid). Thus, it was investigated if esterification (or blocking) of the ionizable carboxyl groups in saccharides was effective for a selective ¹H NMR-aided assay to determine the sulfate content in a mixture of diverse saccharides. As shown in Fig. 5, the esterified galacturonic acid (methyl galacturonate) prevented the galacturonic acid-induced δ change of imidazole, and the addition of NB₄S to the solution recovered the δ value. This suggests that the esterification of saccharides prior to ¹H NMR measurements is a convenient and appropriate pretreatment method to ensure the selectivity of imidazole-guided ¹H NMR assay for determining the sulfate content in saccharides.

Application of ligand-aided ¹H NMR for the estimation of sulfate content of fucoidan

For extensive use of the proposed ligand-aided ¹H NMR for natural sulfated polysaccharides, a typical natural polysaccharide, fucoidan, which is a commercially available chemical, was applied for
the non-destructive method, by comparing the sulfate content assayed by a conventional barium chloride-rhodizonate method. By considering the influence of viscosity and polymer entanglement of polysaccharides at higher concentrations of > 0.1 mg/mL on signal broadening of imidazole protons, the concentration of fucoidan was set to 0.08 mg/mL against different concentrations of imidazole (Figs. 6A and B). As shown in Fig. 6A, no Δδ of imidazole protons (H4,5) was observed up to concentrations of 2.0 mmol-imidazole/g-fucoidan, while at higher concentrations of > 2.0 mmol-imidazole/g-fucoidan, the imidazole proton shifted toward upfield. Notably, the δ value of imidazole proton changed linearly (R²=0.993) in the range of the concentration of 2.0–6.25 mmol-imidazole/g-fucoidan (Fig. 6B). At the inflection point from the regression equation (Fig. 6B), imidazole interacted with sulfate group in fucoidan was estimated to be 0.21 mmol-imidazole/g-fucoidan, indicating that the corresponding sulfate content of fucoidan was 0.21 mmol/g-fucoidan or 20.2 wt%. No repeated NMR experiments were not performed in this study, since δ is not a changeable parameter if NMR measurement conditions are fixed. The sulfate content of the fucoidan was matched with that estimated by a conventional barium chloride-rhodizonate method (20.1 ± 3.2 wt%, n = 3). In addition, the sulfate content estimated by the non-destructive NMR method covered the reported sulfate content in fucoidan estimated by a sulfur-elemental analysis (18.2 wt% and 19.0%). Taken together, the proposed ligand-aided ¹H NMR assay using imidazole can be used for sulfate content of diverse sulfated mono- to polysaccharides without tedious pretreatment (e.g., acid hydrolysis) and with non-destructive preparation.

Conclusions

In this study, ¹H NMR is used for the non-destructive estimation of sulfate content in saccharides using the ligand Δδ values for the first time. Our findings show the imidazole is the most suitable ligand for sulfated saccharides, regardless of the contaminating saccharides, and the sulfate content can be estimated by plotting the observed δ changes as a function of imidazole molar concentration. The proposed ligand-aided ¹H NMR assay can be extensively applied for the non-destructive estimation of
sulfate content in a variety of sulfated saccharides including natural polysaccharides such as fucoidan without tedious pretreatments.

**Conflict of interest**

The authors have declared that no competing interests exist.

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References

1. F. Haroun-Bouhedja, M. Ellouali, C. Sinquin, and C. Boisson-Vidal, *Thromb. Res.*, **2000**, *100*, 453.

2. S. Koyanagi, N. Tanigawa, H. Nakagawa, S. Soeda, and H. Shimeno, *Biochem. Pharmacol.*, **2003**, *65*, 173.

3. M. B. Mansour, R. Balti, L. Yacoubi, V. Ollivier, F. Chaubet, and R. M. Maaroufi, *Int. J. Biol. Macromol.*, **2019**, *121*, 1145.

4. Y. Miyazaki, Y. Iwaihara, J. Bak, H. Nakano, S. Takeuchi, H. Takeuchi, T. Matsui, and D. Tachikawa, *Biochem. Biophys. Res. Commun.*, **2019**, *516*, 245.

5. T. Nishino, Y. Aizu, and T. Nagumo, *Thromb. Res.*, **1991**, *64*, 723.

6. S. You, C. Yang, H. Lee, and B. Y. Lee, *Food Chem.*, **2010**, *119*, 554.

7. S. Palanisamy, M. Vinosha, T. Marudhupandi, P. Rajasekar, and N. M. Prabhu, *Int. J. Biol. Macromol.*, **2017**, *102*, 405.

8. H. Korva, J. Kärkkäinen, K. Lappalainen, and M. Lajunen, *Starch-Stärke*, **2016**, *68*, 854.

9. R. Cao, F. Komura, A. Nonaka, T. Kato, J. Fukumashi, and T. Matsui, *Anal. Sci.*, **2014**, *30*, 383.

10. R. Cao, A. Nonaka, F. Komura, and T. Matsui, *Food Chem.*, **2015**, *171*, 8.

11. I. Berregi, G. del Campo, R. Caracena, and J. I. Miranda, *Talanta*, **2007**, *72*, 1049.

12. H. N. Cheng and T. G. Neiss, *Polym. Rev.*, **2012**, *52*, 81.

13. H. Ihm, S. Yun, H. G. Kim, J. K. Kim, and K. S. Kim, *Org. Lett.*, **2002**, *4*, 2897.

14. K. Sato, T. Onitake, and S. Arai, *Heterocycles*, **2003**, *60*, 779.

15. M. Emami Khansari, M. H. Hasan, C. R. Johnson, N. A. Williams, B. M. Wong, D. R. Powell, R. Tandon, and M. A. Hossain, *ACS Omega*, **2017**, *2*, 9057.

16. C. Jin, M. Zhang, L. Wu, Y. Guan, Y. Pan, J. Jiang, C. Lin, and L. Wang, *Chem. Commun.*, **2013**, *49*, 2025.

17. J. Yoon, S. K. Kim, N. J. Singh, and K. S. Kim, *Chem. Soc. Rev.*, **2006**, *35*, 355.

18. M. Berger and F. P. Schmidtchen, *Angew. Chem. Int. Ed.*, **1998**, *37*, 2694.

19. E. F. Jansen and R. Jang, *J. Am. Chem. Soc.*, **1946**, *68*, 1475.
20. L. J. Silvestri, R. E. Hurst, L. Simpson, and J. M. Settine, *Anal. Biochem.*, 1982, 123, 303.

21. V. Domenici, *Soft Matter*, 2011, 7, 1589.

22. C. Oliveira, A. S. Ferreira, R. Novoa-Carballal, C. Nunes, I. Pashkuleva, N. M. Neves, M. A. Coimbra, R. L. Reis, A. Martins, and T. H. Silva, *Macromol. Biosci.*, 2017, 17, 1600340.

23. M. T. Ale, J. D. Mikkelsen, and A. S. Meyer, *Mar. Drugs*, 2011, 9, 2106.

24. H. Garna, T. H. Emaga, C. Robert, and M. Paquot, *Food Hydrocolloid*, 2011, 25, 1219.

25. M. Rayner, K. Östbring, and J. Purhagen, “*Application of Natural Polymers in Food*”, 2016, Springer International Publishing, Switzerland, 115.

26. K. S. Bittaku, S. Neupane, and S. Alban, *Algal Res.*, 2020, 45, 101759.

27. L. E. Rioux, S. L. Turgeon, and M. Beaulieu, *J. Sci. Food and Agric.*, 2007, 87, 1630.
**Figure Captions**

Fig. 1  Chemical structures of saccharides (galacturonic acid; G6S, galactose-6-O-sulfate; NB4S, neocarrabiose-4-O-sulfate; NTdS, neocarratetraose-41,3-di-O-sulfate) and ligand candidates used in this study.

Fig. 2  Changes in the chemical shifts of 8.3 mM ligand candidates after the addition of 8.3 mM NB4S at a molar ratio of 1:1. $^1$H NMR measurements were carried out at the conditions described in the Experimental section. Arrow indicates a target proton of candidate for complex formation with NB4S.

Fig. 3  Changes in chemical shift ($\delta$) of imidazole protons (H4,5: 7.15 ppm) in the presence of 8.3 mM NB4S as a function of concentration of imidazole (0.83–25 mM: molar ratio of 0.2:1–3:1) (A). A plot of chemical shift ($\delta$) against molar ratio (B).

Fig. 4  Changes in chemical shift of imidazole protons (H4,5) in the presence of non-sulfated and sulfated oligosaccharides. A molar ratio of imidazole against each saccharide was 1:1. Saccharides used in this study were D-(+)-galactose (Gal), 3$\beta$-galactobiose (GalB), and 3$\alpha$-4$\beta$-3$\alpha$-galactotetraose (GalT) as non-sulfated saccharides, and galactose-6-O-sulfate (G6S), neocarrabiose-4-O-sulfate (NB4S), and neocarratetraose-41,3-di-O-sulfate (NTdS) as their corresponding sulfated saccharides.

Fig. 5  Effect of carboxylated saccharide, galacturonic acid, on chemical shift of imidazole protons (H4,5). Methyl galacturonate (methyl-$\alpha$-D-galacturonate), an esterified galacturonic acid, was used as decarboxylated saccharide. Esterification of galacturonic acid was performed as described in the Experimental section. NB4S was used as sulfated saccharide. A molar ratio of imidazole against each saccharide was 1:1.

Fig. 6  Changes in chemical shift of imidazole protons (H4,5) in the presence of *Fucus vesiculosus* fucoidan (Fuc, 0.08 mg/mL) as a function of concentration of imidazole (1.25–6.25 mmol-imidazole/g-Fuc) (A). A plot of chemical shift ($\delta$) against concentration of imidazole (B).
Fig. 1

Saccharides

Galacturonic acid
(non-sulfated carboxylated monosaccharide)

G6S
(sulfated monosaccharide)

NB4S
(sulfated disaccharide)

NTdS
(disulfated tetrasaccharide)

Ligand candidates

Imidazole

L-Arginine

Piperazine

Glycine

L-(2-Pyrimidinyl) piperazine
Fig. 2
Fig. 3

(A) Imidazole

| Ratio | δ (ppm) |
|-------|---------|
| 0.2:1 |         |
| 0.5:1 |         |
| 0.8:1 |         |
| 0.9:1 |         |
| 1:1   |         |
| 1.1:1 |         |
| 1.2:1 |         |
| 1.5:1 |         |
| 2:1   |         |
| 3:1   |         |

B) Imidazole δ (ppm) vs. [imidazole]/[sulfated saccharide]
Imidazole alone

Imidazole + Gal

Imidazole + Gal + G6S

Imidazole + GalB

Imidazole + GalB + NB4S

Imidazole + GalT

Imidazole + GalT + NTdS

δ (ppm)

Fig. 4
Fig. 5
Fig. 6

(A)  
1.25 mmol-imidazole/g-Fuc
1.75 mmol/g
2.0 mmol/g
2.25 mmol/g
2.5 mmol/g
2.88 mmol/g
3.0 mmol/g
3.75 mmol/g
6.25 mmol/g

\[ \delta (\text{ppm}) \]

(B)  
\[ y = -0.3067x + 7.5448 \]
\[ R^2 = 0.993 \]

\[ \text{Imidazole } \delta (\text{ppm}) \]

\[ \text{mmol-imidazole/g-Fuc} \]
Graphical Index

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