Supplementary Information

Heterocyclic Boronic Acids Display Sialic Acids Selective Binding under Hypoxic Tumor Relevant Acidic Environment.

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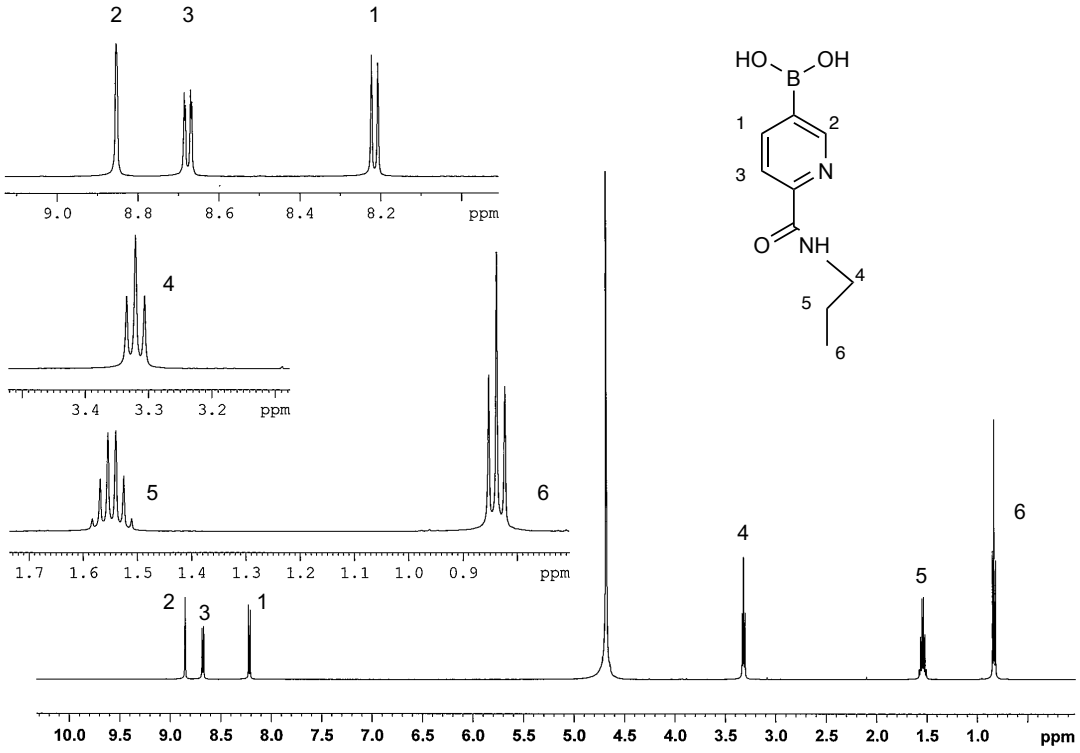
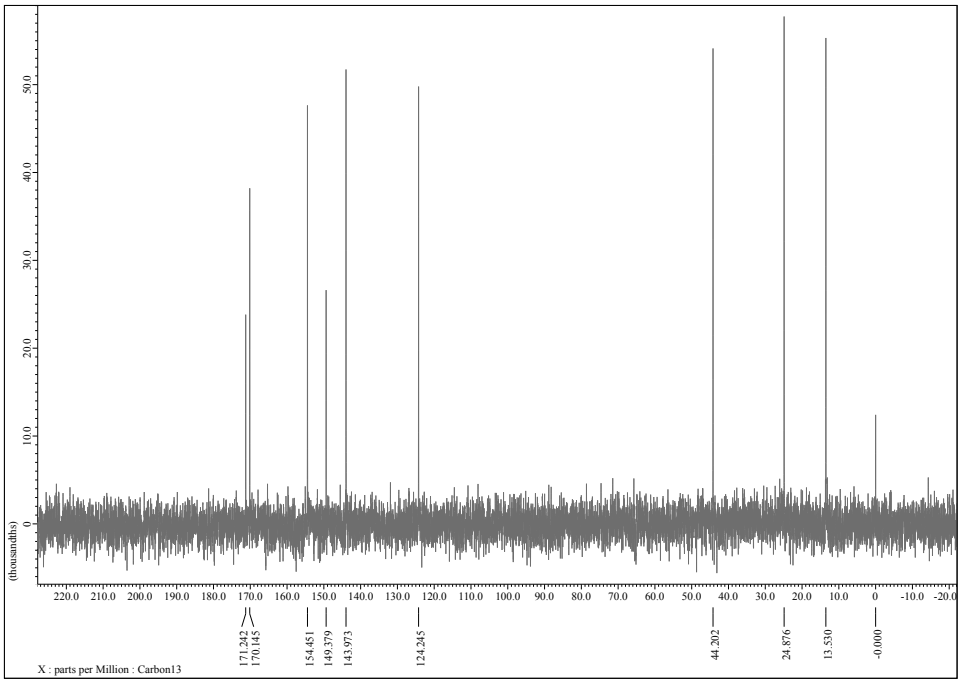
Experimental

Materials and methods. 3-Pyridylboronic acid (Wako Pure Chemical Industries), 5-pyrimidine boronic acid (Wako Pure Chemical Industries), 6-methoxypyridin-3-boronic acid (Wako Pure Chemical Industries), 2-fluoro-3-pyrimidin boronic acid (Wako Pure Chemical Industries), pyrazole-4-boronic acid (Sigma-Aldrich), pyrazole-5-boronic acid (Sigma-Aldrich), 5-boronopicolinic (Santa Cruz Biotechnology)and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride n-hydrate (DMT-MM) (Wako Pure Chemical Industries) and N-(6-Aminohexyl)rhodamine 6G-amide bis(trifluoroacetate) (Sigma-Aldrich) were all used without further purification. 3-Borono-1-(carboxymethyl)pyridine was synthesized by a method previously reported.[8c]. All sugars (N-acetylneuraminic acid, glucose, galactose, mannose, fucose and xylose) were purchased from Wako Pure Chemical Industries and used without further purification.

All ^{1}H nuclear magnetic resonance (NMR) spectra were taken on a Bruker 500MHz spectrometer and all mass spectra (high-resolution electrospray ionization mass spectrometry: HRESIMS) were collected and analyzed on a Bruker microTOF spectrometer.

Synthesis of (6-propylcarbamoyl)pyridine-3-)boronic acid. To a solution of 5-boronopicolinic acid (500 mg, 3 mmol) and propylamine (200 mg, 3.1 mmol) in DMF (30 mL) was added DMT-MM (1.2 g, ca. 4 mmol). The mixture was stirred at r. t. overnight. The solvent was removed and the crude yellow oil was dissolved in aq. 0.1 M HCl (10 mL/mmol) and then evaporated by rotary evaporator. The resulting light-yellow colored solid was then stirred in acetone to give white crystalline precipitate (660 mg, 94%); ^{1}H NMR (D_2O, 400 MHz): δ 8.86 (s, 1H), 8.68 (d, 1H), 8.21 (d, 1H), 3.32 (t, 2H), 1.55 (sext, 2H), 0.84 (t, 3H); ^{13}C NMR (D_2O, 96 MHz): δ 171.24, 170.15, 154.45, 149.38, 143.97, 124.25, 44.20, 24.88, 13.53; HRESIMS *m/z* calculated for $C_9H_{14}BN_2O_3^+$ ($M + H^+$) 209.11, found 209.08.

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40.0
30.0
20.0
10.0
0
220.0 210.0 200.0 190.0 180.0 170.0 160.0 150.0 140.0 130.0 120.0 110.0 100.0 90.0 80.0 70.0 60.0 50.0 40.0 30.0 20.0 10.0 0 -10.0 -20.0
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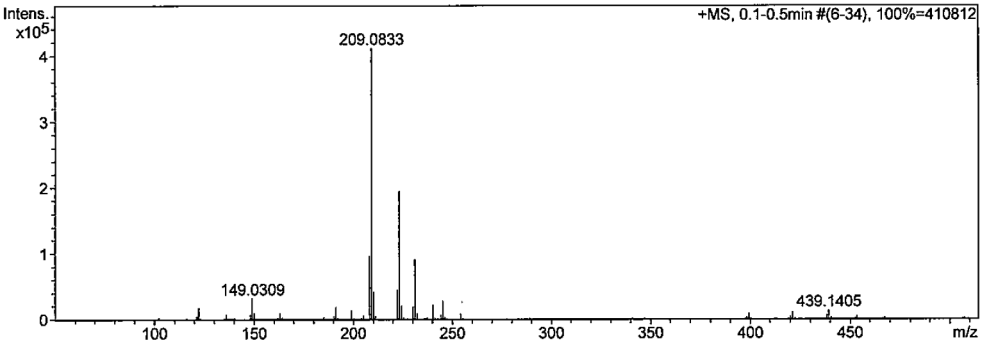
154.451
149.379
143.973
124.245
44.202
24.876
13.530
-0.000
X : parts per Million : Carbon13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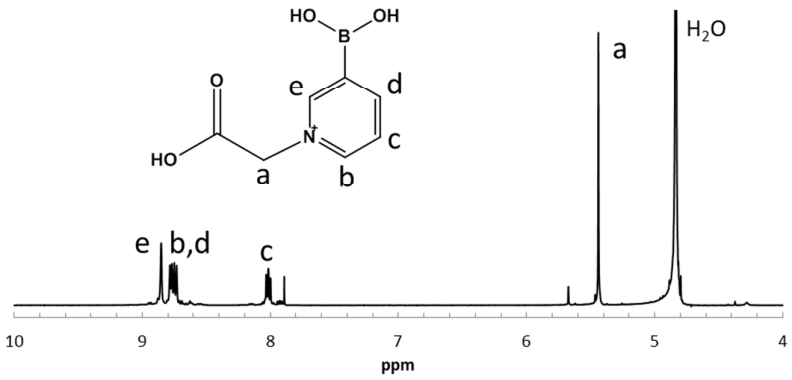
x10^5
+MS, 0.1-0.5min #(6-34), 100%=410812
209.0833
149.0309
439.1405
m/z

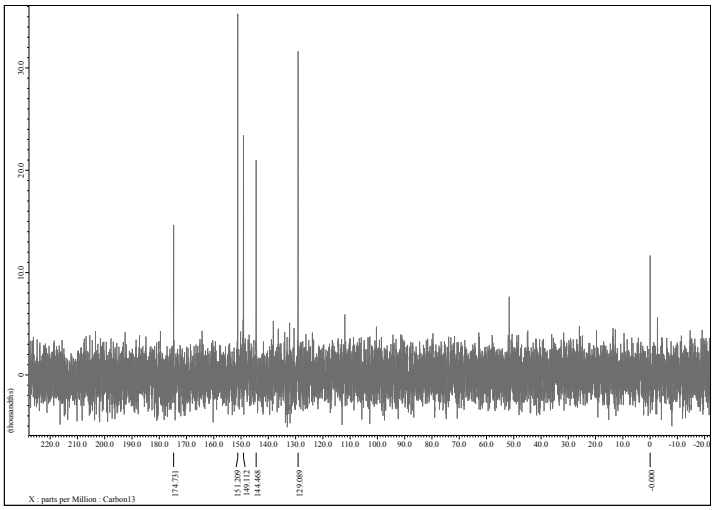
Identification of 3-Borono-1-(carboxymethyl)pyridine.

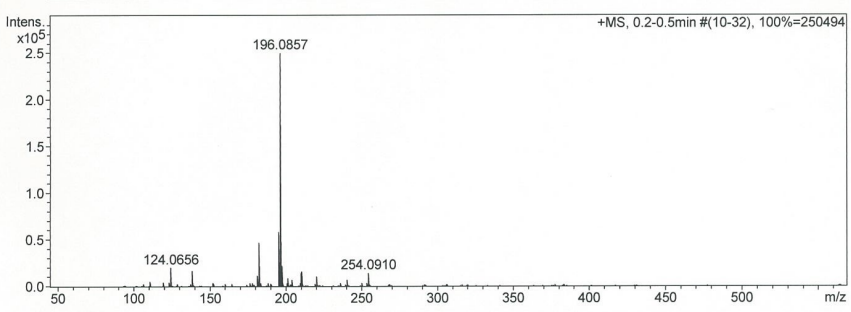
^{1}H NMR (D_2O, 400 MHz): δ 8.83 (d, 1H), 8.79-8.75 (m, 2H), 8.03 (t, 1H), 5.42 (s, 1H);

^{13}C NMR (D_2O, 400 MHz): δ 174.73, 151.21, 149.11, 144.47, 129.09, 112.02, 51.74;

HRESIMS *m/z* calculated for $C_8H_{12}BNO_4^-$ (M + OH^-) 196.09, found 196.09.

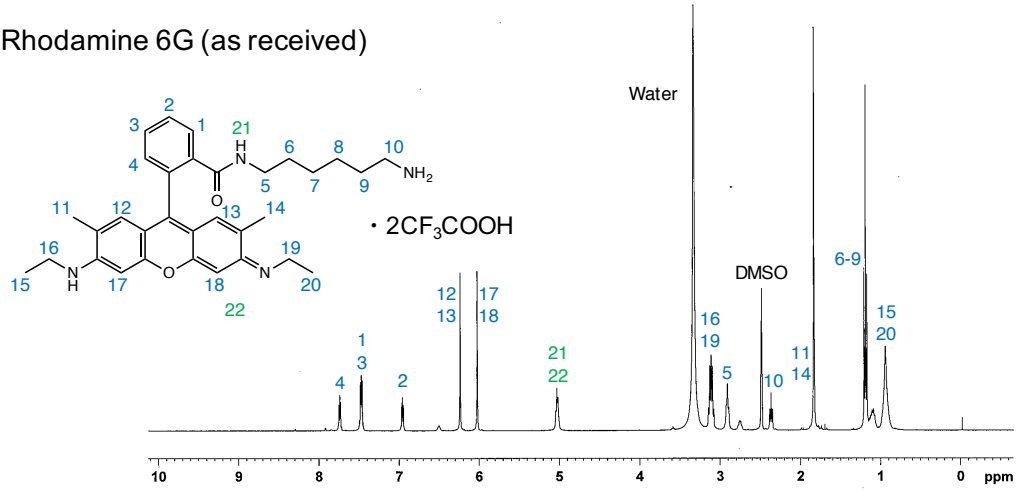




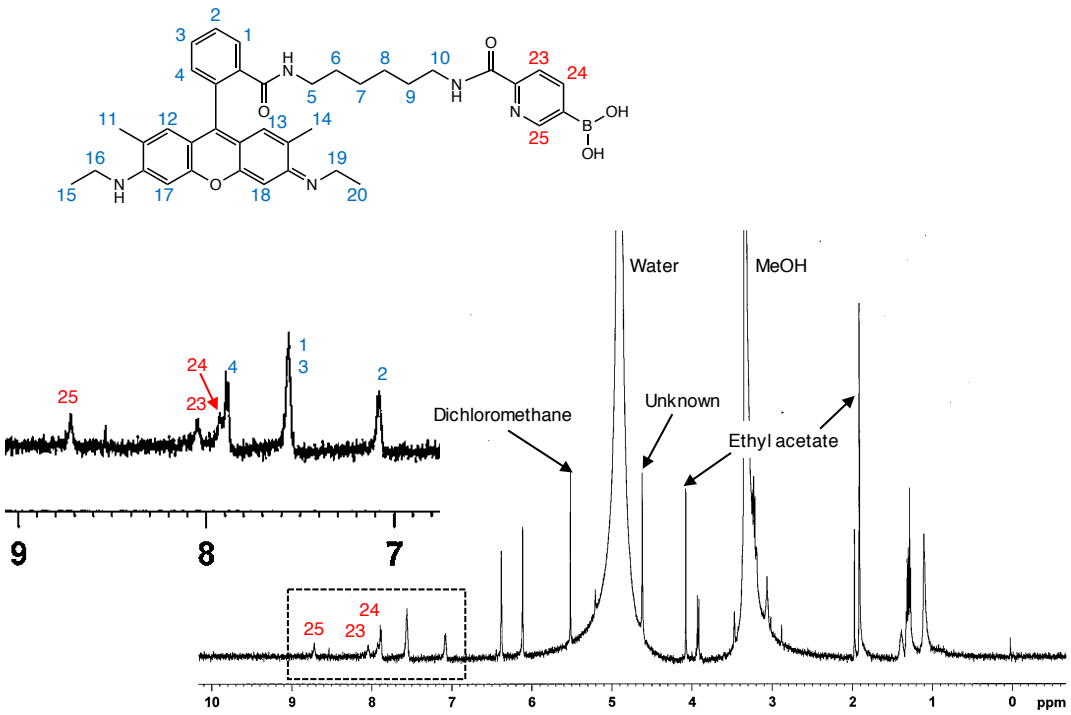


Rhodamine conjugation of 5-boronopicolinic acid. To a solution of 5-boronopicolinic acid (15 mg, 90 μmol) and N-(6-Aminohexyl)rhodamine 6G-amide bis(trifluoroacetate) (50 mg, 70 μmol) in DMF (10 mL) was added DMT-MM (30 mg, ca. 100 μmol) and triethylamine (10 mg, 100 μmol). The mixture was stirred at r. t. for 3 hours. The solvent was removed and the crude was dispersed in chloroform and washed with brine several times. The organic layer was evaporated and dried in vaccuo to yield red solid (55 mg, 89 %); ^{1}H NMR (MeOD 400 MHz): δ 8.74 (s, 1H), δ 8.03 (d, 1H), δ 7.92 (d, 1H), δ 7.89 (t, 1H), δ 7.55 (t, 2H), δ 7.07 (t, 1H), δ 6.23 (s, 2H), δ 6.03 (s, 2H), δ 3.11 (t, 4H), δ 1.95 (s, 6H), δ 1.26 (t, 8H), δ 1.10 (s, 6H); HRESIMS *m/z* calculated for $C_{40}H_{51}BN_7O_5^+$ $(M + NH_4 + CH_3CN)^+$ 720.40, found 720.43. The yield of modification was calculated to be 84 % based on the peak area integration ratio: 23-24 *vs.* 1-4 on the chart.

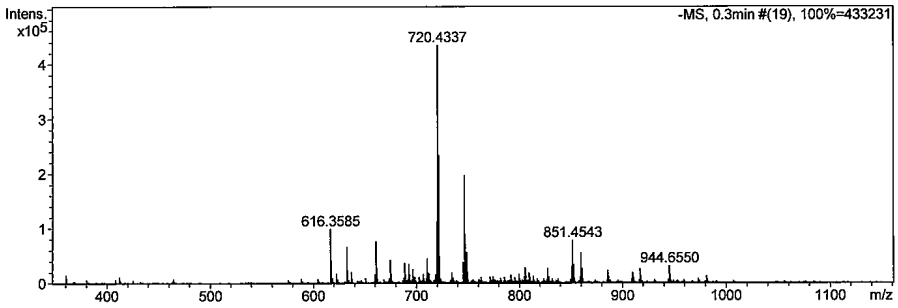
A: Rhodamine 6G (as received)



B: Rhodamine conjugate with 5-boronopicolinic acid



C: HRESIMS spectrum of B



Boron Nuclear Magnetic Resonance (^{11}BNMR). Sample solutions for NMR were prepared by dissolving both sugars and boronic acid compounds in deionized-distilled water under stirring at room temperature. The concentration of each compound was 20mM unless otherwise specified. The pH of the solution was adjusted with hydrochloric acid and sodium hydroxide. As soon as the solutions were prepared, the NMR spectra were recorded. The B^{11} NMR spectra were collected with JEOL EX 300 spectrometer at 96 MHz, the chemical shifts of which were referenced to that of boron trifluoride-diethyl etherate (0 ppm). The measurements were carried out in a tube made of poly(tetrafluoroethylene) in order to avoid any appearance of noise due to boron impurity incorporated in a glass. Data was processed using Delta NMR Processing and control software, version 5.0.4.4.

^{11}B NMR is commonly used to elucidate boronic acid–diol interactions where the observed chemical shift is sensitive to hybridization state of the boron nucleus, i.e., trigonal or tetrahedral. (Duin, M. V.; Peters, J. A.; Kieboom, A. P. G.; Bekkum, H. V. *Tetrahedron* **1984**, 40, 2901 / Berg, R. V. D.; Peters, J. A.; Bekkum, H. V. *Carbohydr. Res.* **1994**, 253, 1) Typically, the tetrahedral state or boronate displays the chemical shifts at around 0 ppm, whereas the trigonal state (boronic acid) results in a significant downfield resonance. In the absence of binding targets, due to a rapid interconversion between the two hybridization states, only one signal appears at a field corresponding to the ratio of the two states at a given pH; as a result, the chemical shift undergoes a downfield shift with decrease of the pH over the range of the pK_a value of boronic acid of interest. When allowed to complex with diols, since the rate of exchange between the free state and the complex state is now slow relative to the ^{11}B NMR time scale, the spectrum consists of two peaks: a free boronic acid/boronate peak, whose chemical shift shows a downfield shift with decrease of the pH similar to the above, and a peak attributable to the complex at a higher field (around 0 ppm). Assignment was made to each peak on the spectra for a series of pHs. When necessary, overlapped peaks were deconvoluted before analysis using the above-mentioned software.

Stability constants were calculated by integrating the peak area assigned to the complex and the free state and fitting them to the following equation (where all values are known due to the the initial concentration of sugars and boronics acid being identical, i.e.,

20mM):

$$K_{Stability} = \frac{[BA{:}\,Diol_{complex}]}{[BA_{Free}][Diol_{Free}]}$$

For some derivatives displayed in Figure 1 (5-pyrimidine boronic acid, pyrazole-5-bprpnic acid and 5-boronopicolinic) whose pKa values were not available in literature, B^{11} NMR titration was carried out. Namely, each centroid value of chemical shifts was plotted for various pHs to obtain the titration curve and its inflection point was defined as the pKa (SI TABLE 1).

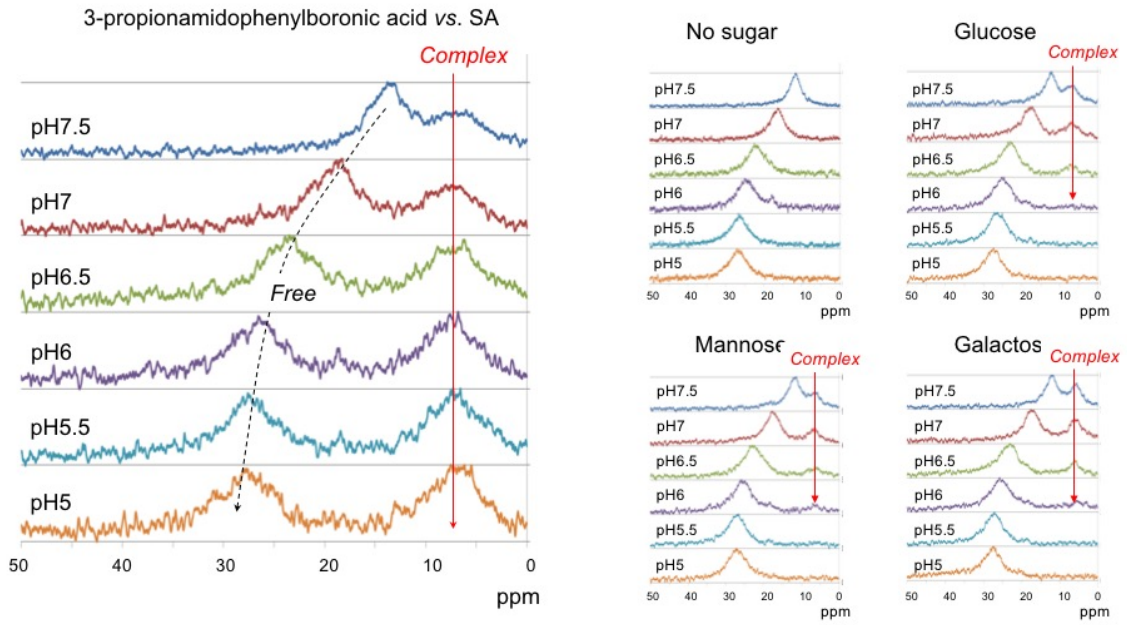
ARS Assay. Alizarin Red S (ARS) (Wako Pure Chemical Industries) was used as received. The water used for the binding studies was double distilled and further purified with a Milli-Q filtration system. A fluorimeter (Tecan Microplate Reader: Tecan infinite 200) was used for all fluorescent and absorbance studies, which were conducted in a 96 well plate format (SI FIGURE 14). The method and data analysis was as outlined by Springsteen and Wang[9a] unless otherwise stated.

In vitro assay. Human pancreatic epithelioid carcinoma cell (PANC1) was used for the assay. 250K cells/ml were plated on Iwaki 35mm glass base plates and incubated for 48 hours at 37°C. Experiments were done in pH 7.4, physiological pH and pH 6.5, which is the lowest pH found inside tumor. Cells were first stained with 10mg/ml Hoechst (PromoCell GmbH) and then incubated for 20 minutes with 200µg/ml of each boronic acid per plate at room temperature and finally stained with 100µg/ml per plate Fluorescein–labeled *Sambucus Nigra* (SNA, EBL) lectin (Vector Laboratories, Inc.) for 25 minutes at 4° C. Control plates were stained with only Hoechst and the SNA-lectin. Finally, fluorescence intensity was measured and calculated by using confocal laser scanning microscopy and imageJ software. Specificity study using Rhodamine conjugate of 5-boronopicolinic acid was performed as follows. 20K cells/200 µl were plated on Chambered # 1.0 Borosilicate Coverglass system (Thermo Scientific). Cells were incubated for 48 hours and then stained with Hoechst. After washing, the media was replaced by PBS (pH 6.5 and pH 7.4; 190µl). Cells were then incubated for 15 minutes at 4°C with 10µl of the rhodamine conjugate dissolved in DMSO (200 µg/ml). Cells were again washed with PBS (pH 6.5 and pH 7.4) before imaging. A negative

control experiment was carried out by incubating the cells with 1µl of sialidase (Neuraminidase (1U/50 µl 25mM Tris buffer pH 7.5) from *Clostridium perfringens;* Roche Diagnostics GmbH) for 40 minutes at 37°C. Cells were then washed, stained with Hoechst and finally incubated with the rhodamine conjugate and analyzed in the same manner as above.

Glycan array. Glycan array experiment was carried out on the basis of commercial analytical service at Sumitomo Bakelite Co., Ltd. by using its product BS-X 1733Z (Type I: 28 glycans). Structural details of 28 different glycans immobilized on each well are summarized in SI FIGURE 15. Biotinylated *Sambucus Nigra* (SNA, EBL) lectin (Vector Laboratories, Inc.) and Biotinylated *Wheat Germ Agglutinin* (WGA) lectin (Vector Laboratories, Inc.) were conjugated with Cy3-labled Streptavidin (Sigma-Aldrich) using a commercial labeling kit: HiLyte FluorTM 555 Labeling Kit-NH2 (Dojindo Molecular Technology, Inc.). For specificity test, 1 µg/mL Cy3-labeled SNA lectin and 100 µg/mL µg/mL Cy3-labled WGA lectin were applied to the array and compared (SI FIGURE 16). For competition assay, wells were first equilibrated with 70 µL PBS solutions (conditioned either at pH 7.4 or 6.5) with and without 4 mM 5-boronopicolinic acid for 20 minutes at room temperature. Thereafter, a half (35 µL) volume of each well was exchanged by the same volume solution containing 100 ng/mL Cy3-labeled SNA. The reaction was allowed for 90 minutes at room temperature before optical analysis on evanescent light scanner instrument (BIO-REX SCAN 200 (Rexxam Co., Ltd.)) (SI FIGURE 17).

A



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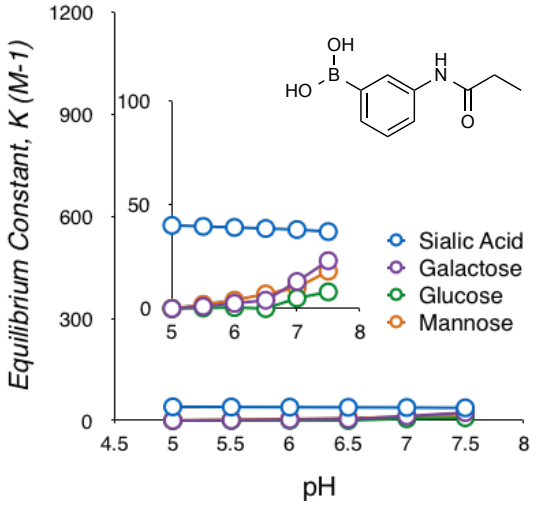
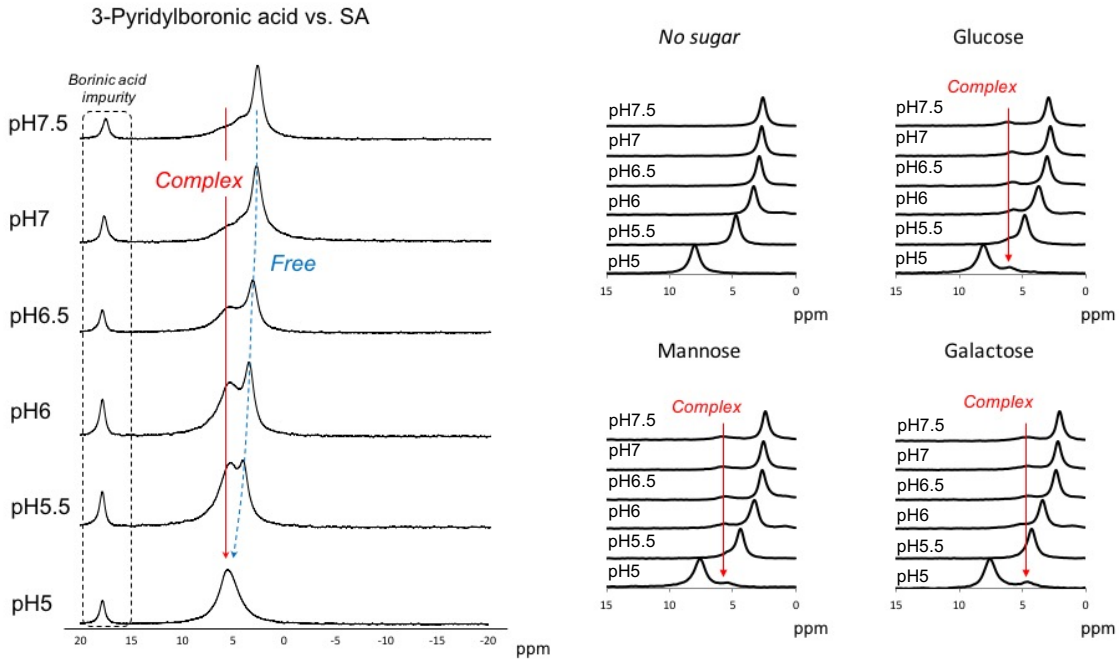


SI FIGURE 1.

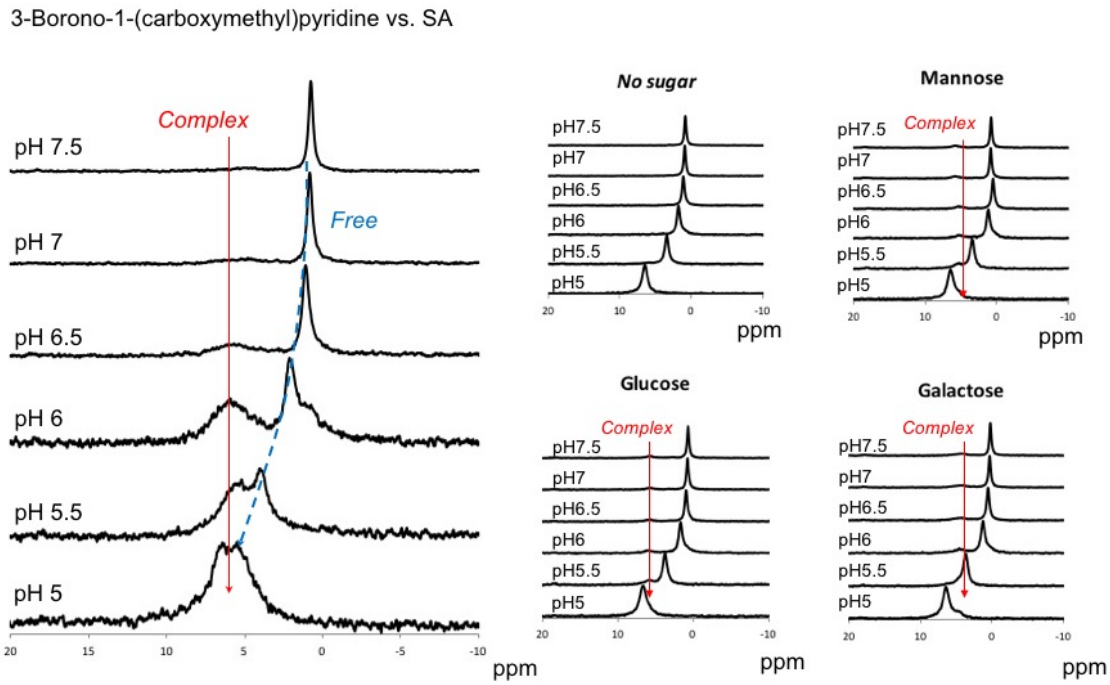
(A) ^{11}B NMR spectra of aqueous solutions of 3-propionamidophenylboronic acid with a variety of sugars (20mM/20mM) for various pHs. (B) Binding constants (*K*) between 3-propionamidophenylboronic acid and a variety of sugars, as a function of pH.



SI FIGURE 2.

^{11}B NMR spectra of aqueous solutions of 3-pyridylboronic acid with a variety of sugars (20mM/20mM) for a series of pHs.

A



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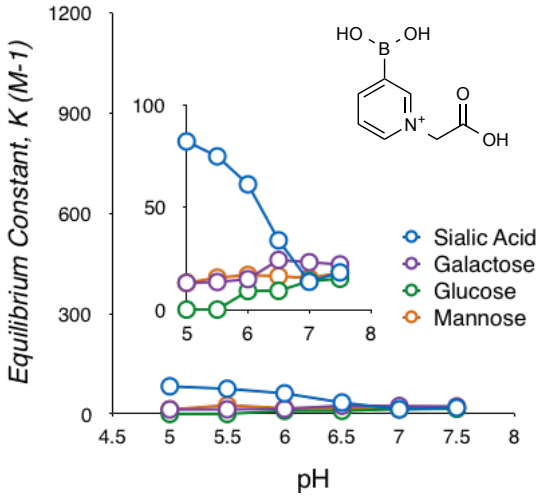
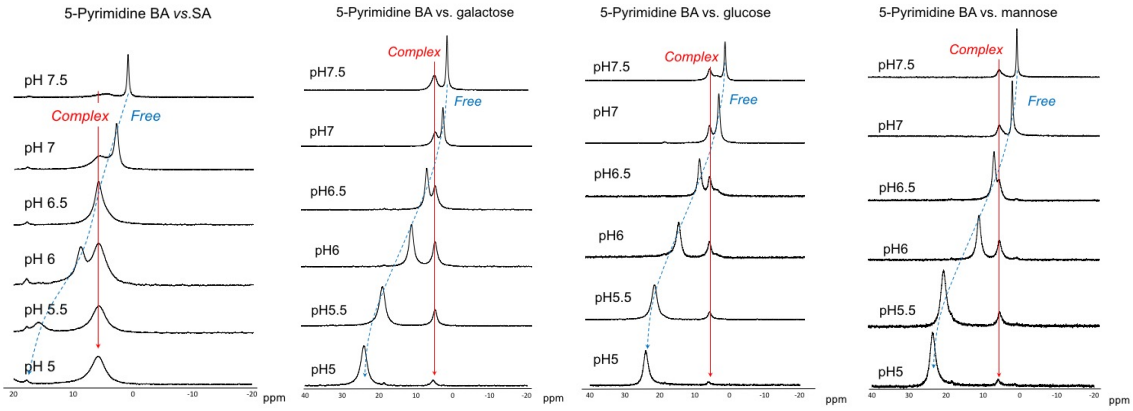


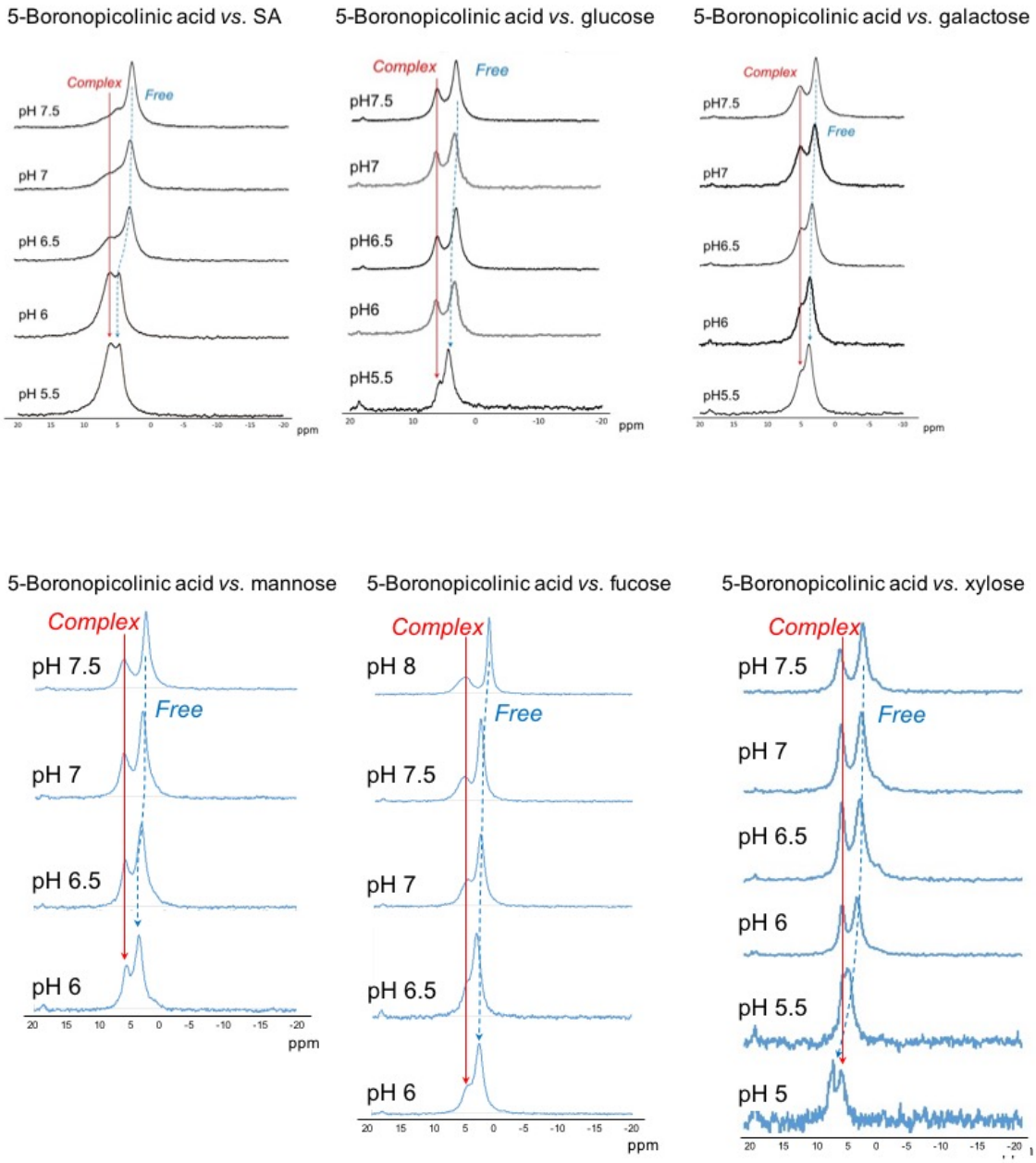
SI FIGURE 3.

(A) ^{11}B NMR spectra of aqueous solutions of 3-borono-1-(carboxymethyl)pyridine with a variety of sugars (20mM/20mM) for a series of pHs. (B) Binding constants (*K*) between 3-borono-1-(carboxymethyl)pyridine and a variety of sugars, as a function of pH.



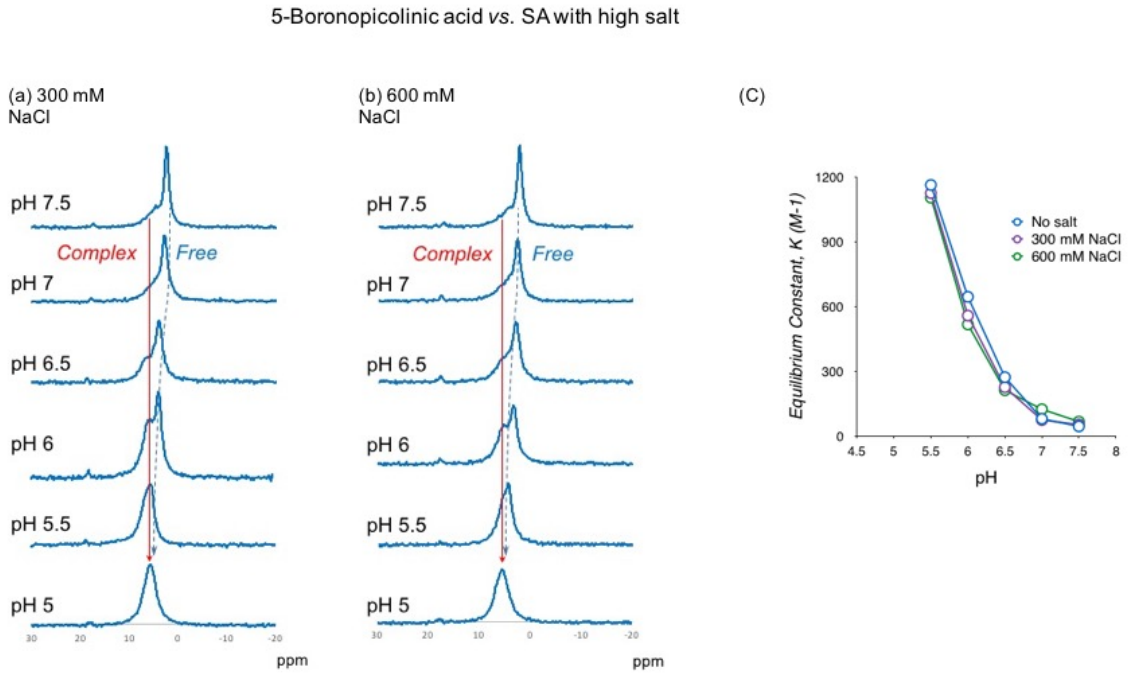
SI FIGURE 4.

^{11}B NMR spectra of aqueous solutions of 5-pyrimidine boronic acid with a variety of sugars (20mM/20mM) for a series of pHs.



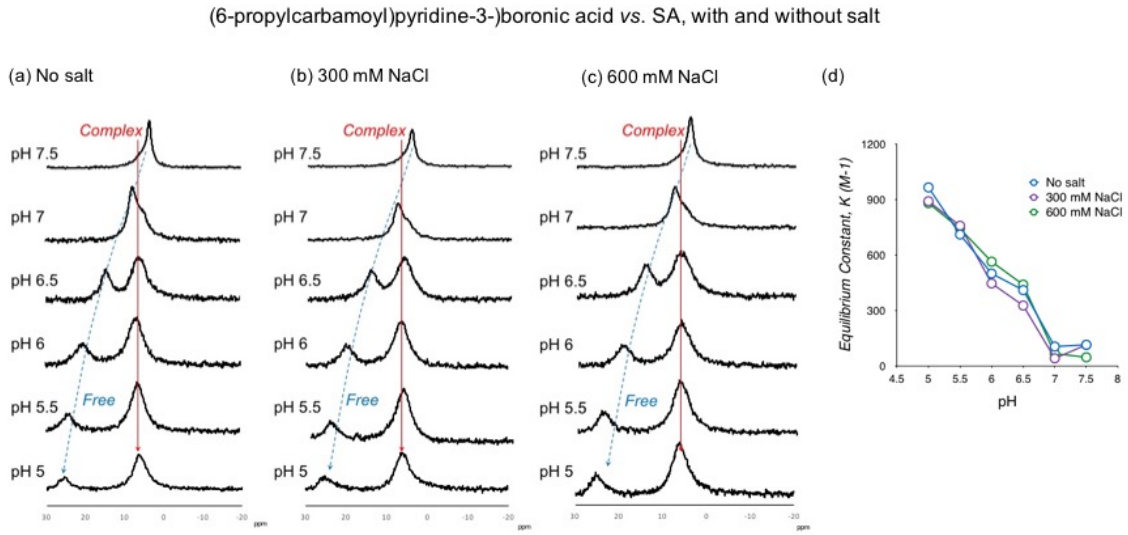
SI FIGURE 5.

^{11}B NMR spectra of aqueous solutions of 5-boronopicolinic acid with a variety of sugars (20mM/20mM) for a series of pHs.



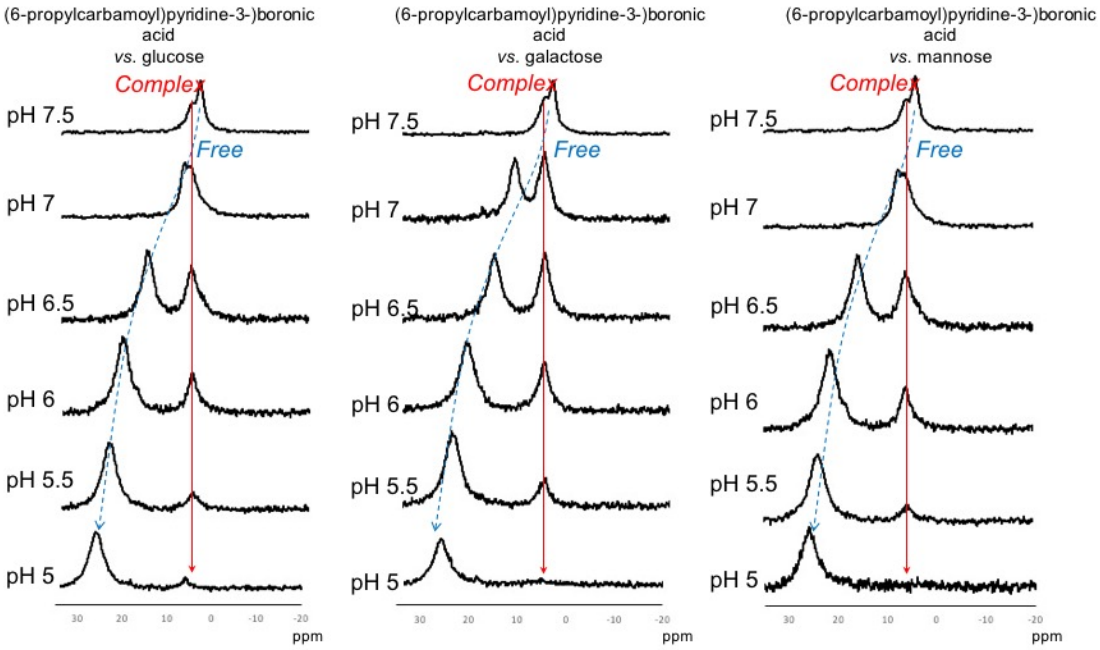
SI FIGURE 6.

(a), (b) ^{11}B NMR spectra of 20 mM aqueous solutions of 5-boronopicolinic acid containing 20mM SA for different sodium chloride concentrations (300 mM and 600 mM, respectively) and pHs. (c) Binding constants between 5-boronopicolinic acid and SA as a function of pH for different salt concentrations.



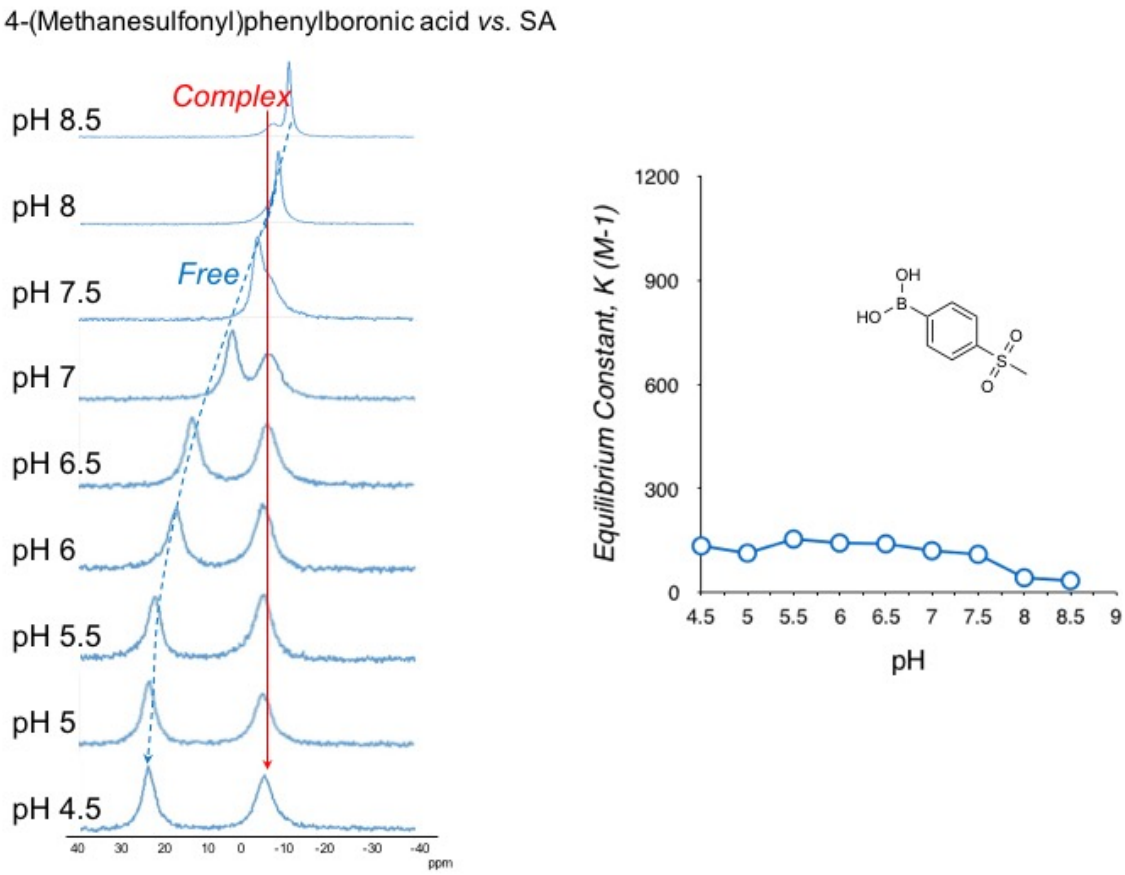
SI FIGURE 7.

(a), (b), (c) ^{11}B NMR spectra of 20 mM aqueous solutions of (6-propylcarbamoyl)pyridine-3-)boronic acid containing 20mM SA for different sodium chloride concentrations (0 mM, 300 mM and 600 mM, respectively) and pHs. (d) Binding constants between (6-propylcarbamoyl)pyridine-3-)boronic acid and SA as a function of pH for different salt concentrations.



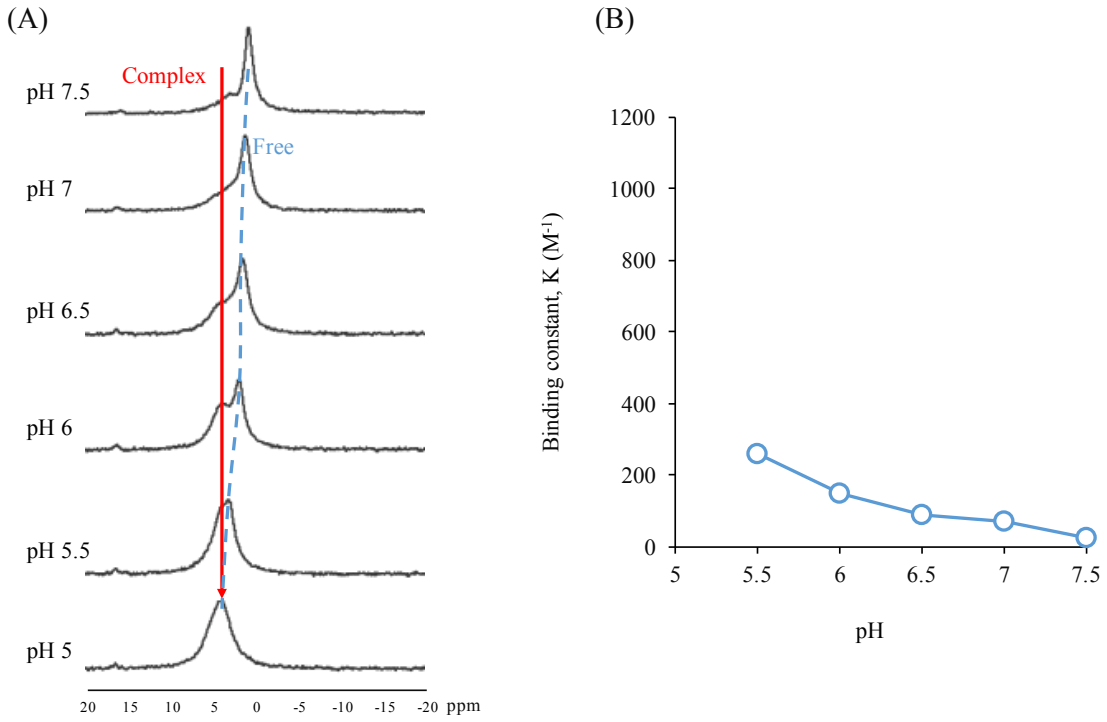
SI FIGURE 8.

^{11}B NMR spectra of aqueous solutions of (6-propylcarbamoyl)pyridine-3-)boronic acid with a variety of sugars (20mM/20mM) for a series of pHs.



SI FIGURE 9.

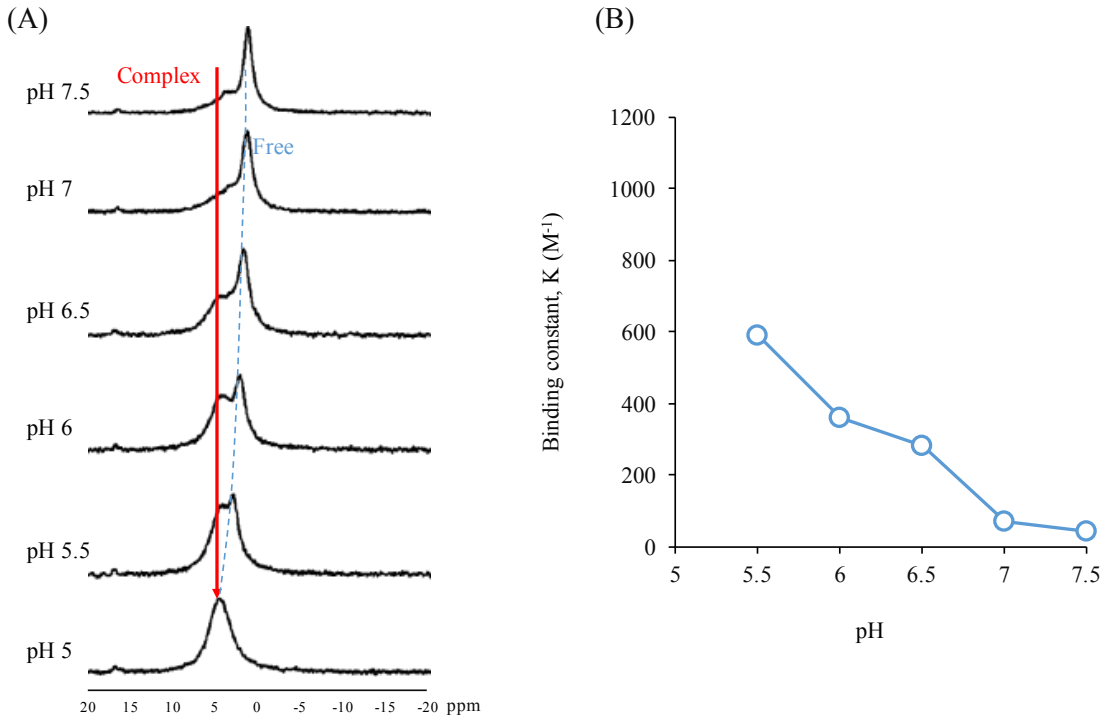
(*Left*) ^{11}B NMR spectra of aqueous solutions of 4-(methylsulfonyl)benzeneboronic acid containgin SA (20mM/20mM) for a series of pHs. (*Right*) Binding constants between 4-(methylsulfonyl)benzeneboronic acid and SA as a function of pH for different salt concentrations.



SI FIGURE 10.

(A) ^{11}B NMR spectra of aqueous solutions of 5-boronopicolinic acid with *N*-acetylneuraminic acid methyl ester (20mM/20mM) for a series of pHs.

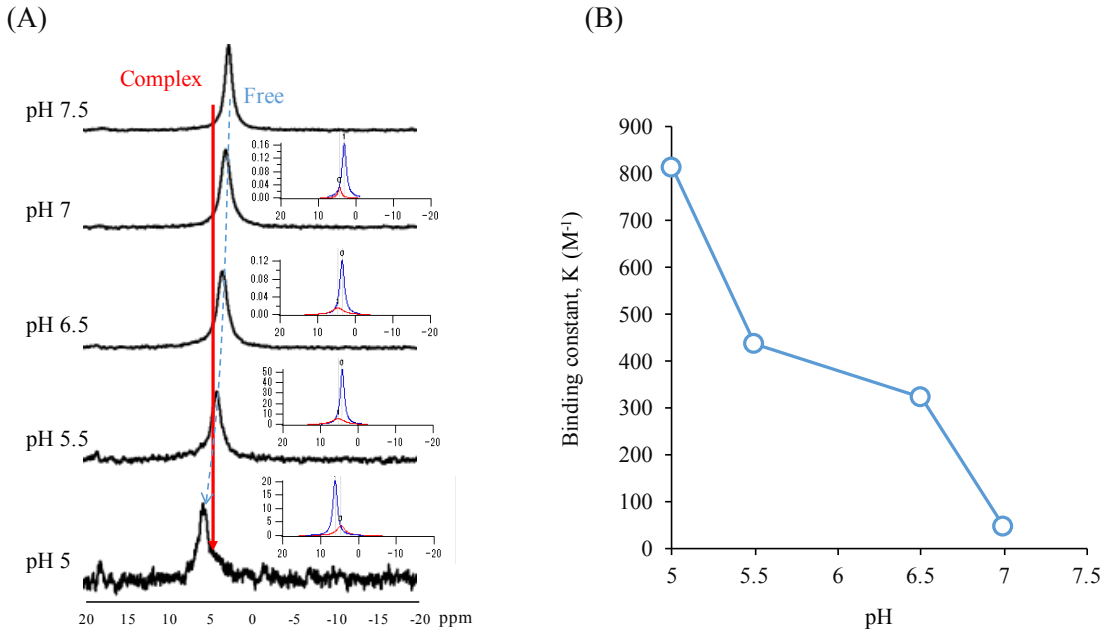
(B) Binding constants between 5-boronopicolinic acid and N-acetylneuraminic acid methyl ester as a function of pH.



SI FIGURE 11.

(A) ^{11}B NMR spectra of aqueous solutions of 5-boronopicolinic acid with a N-glycolylneuraminic acid (20mM/20mM) for a series of pHs.

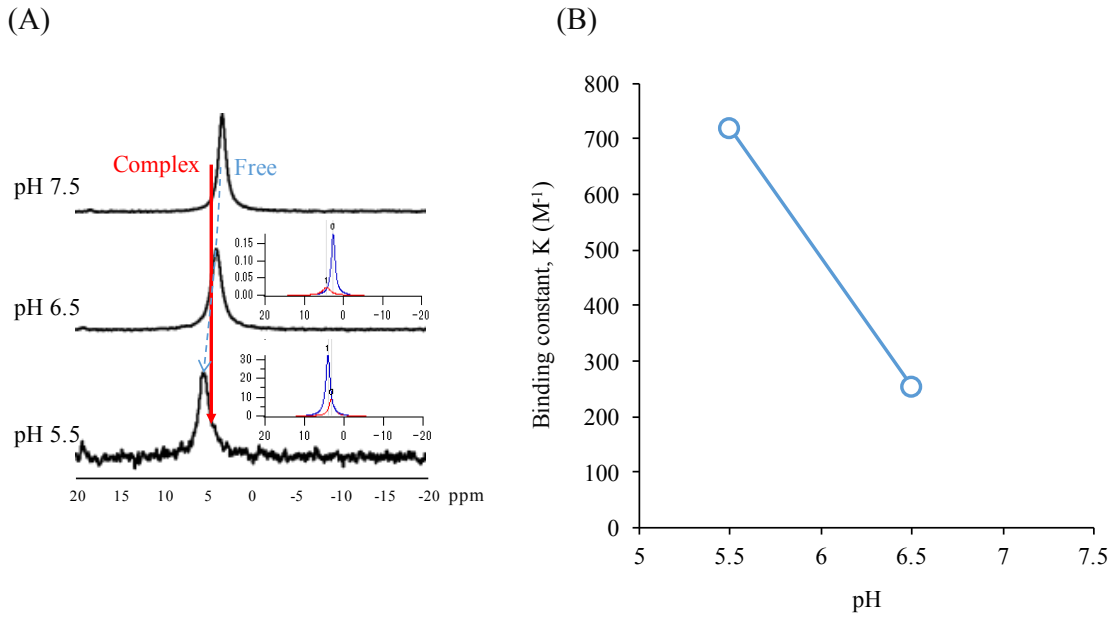
(B) Binding constants between 5-boronopicolinic acid and N-glycolylneuraminic acid as a function of pH.



SI FIGURE 12.

(A) ^{11}B NMR spectra of aqueous solutions of 5-boronopicolinic acid with a N-acetylneuraminic acid trimer (α , 2→8) (20mM/5.7mM) for a series of pHs.

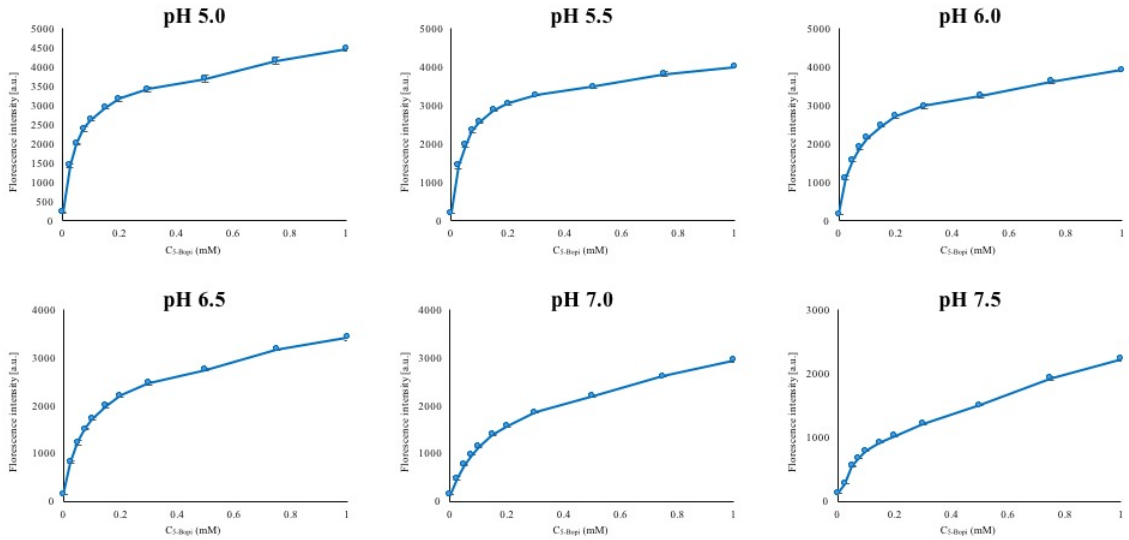
(B) Binding constants between 5-boronopicolinic acid and N-acetylneuraminic acid trimer (α , 2→8) as a function of pH.



SI FIGURE 13.

(A) ^{11}B NMR spectra of aqueous solutions of 5-boronopicolinic acid with a N-acetylneuraminic acid pentamer (α , 2→8) (20mM/5.3mM) for a series of pHs.

(B) Binding constants between 5-boronopicolinic acid and N-acetylneuraminic acid pentamer (α , 2→8) as a function of pH.



SI FIGURE 14-1.

Florescence intensity (ex: 468 nm, em: 572 nm) changes of 9 uM Alizarin red S (ARS) aqueous solutions containing different amount of 5-boronopicolinic acid (5-Bopi) for various pHs. Data are presented as means ± standard deviation (SD), (n=3).

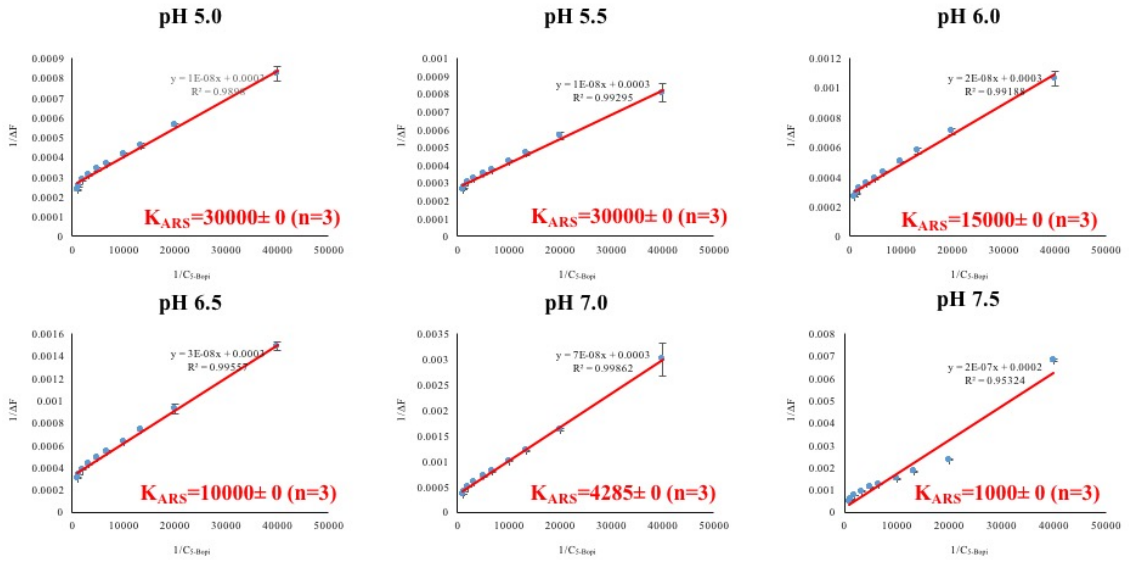
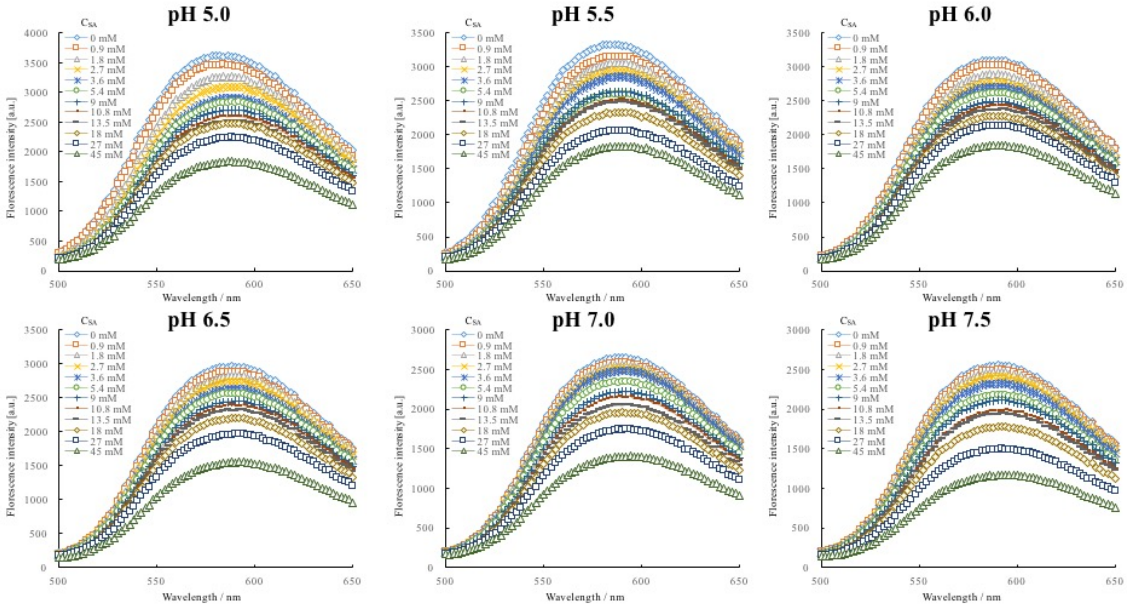


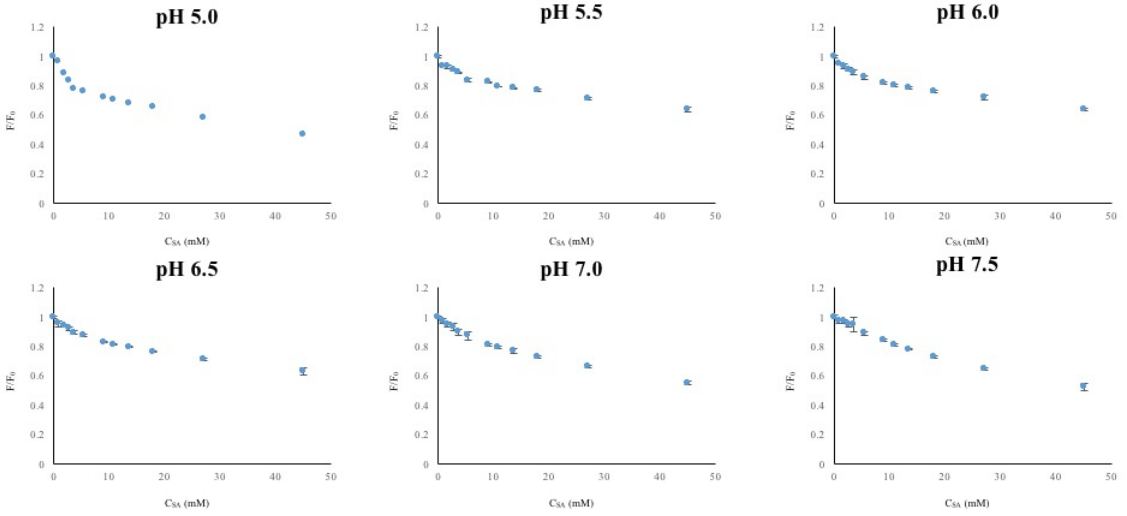
Figure 14-2

Binding constant of 5-Bopi and ARS (K_{ARS}). K_{ARS} is the quotient of the intercept and the slope in plots of 1/Δ Florescence intensity (ΔF) versus $1/C_{5\text{-Bopi}}$. Data are presented as means ± SD, (n=3).



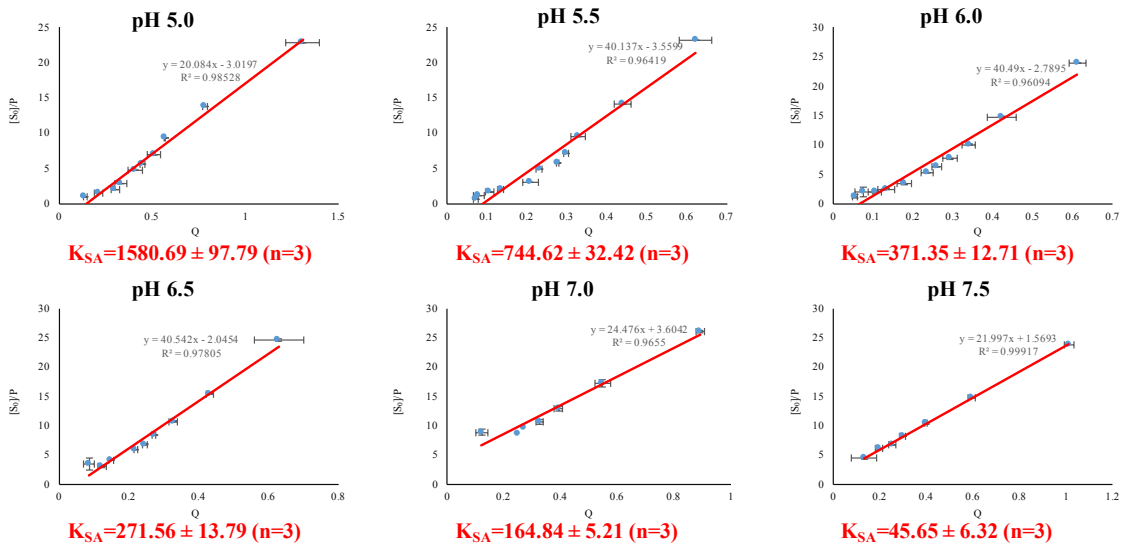
SI FIGURE 14-3.

Fluorescence spectra (ex: 468 nm) of ARS-5-Bopi complex solutions for various concentration of SA (C_{SA}) and pHs.



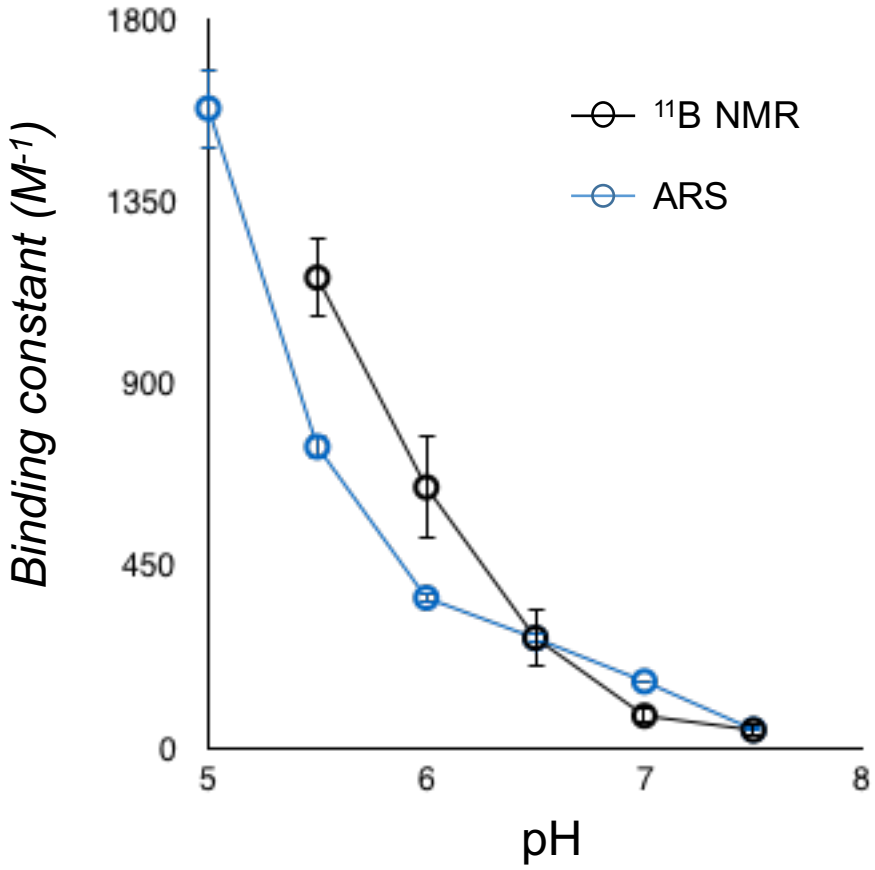
SI FIGURE 14-4.

Titration of sialic acid (SA) into a solution of ARS- 5-Bopi complex. Decrease of florescent intensity (ex: 468 nm, em: 572 nm) of ARS- 5-Bopi complex by the presence of SA; F_0 initial fluorescent intensity of solution, F fluorescent intensity of solution, and C_{SA} concentration of SA. Data were presented as means ± SD, (n=3).



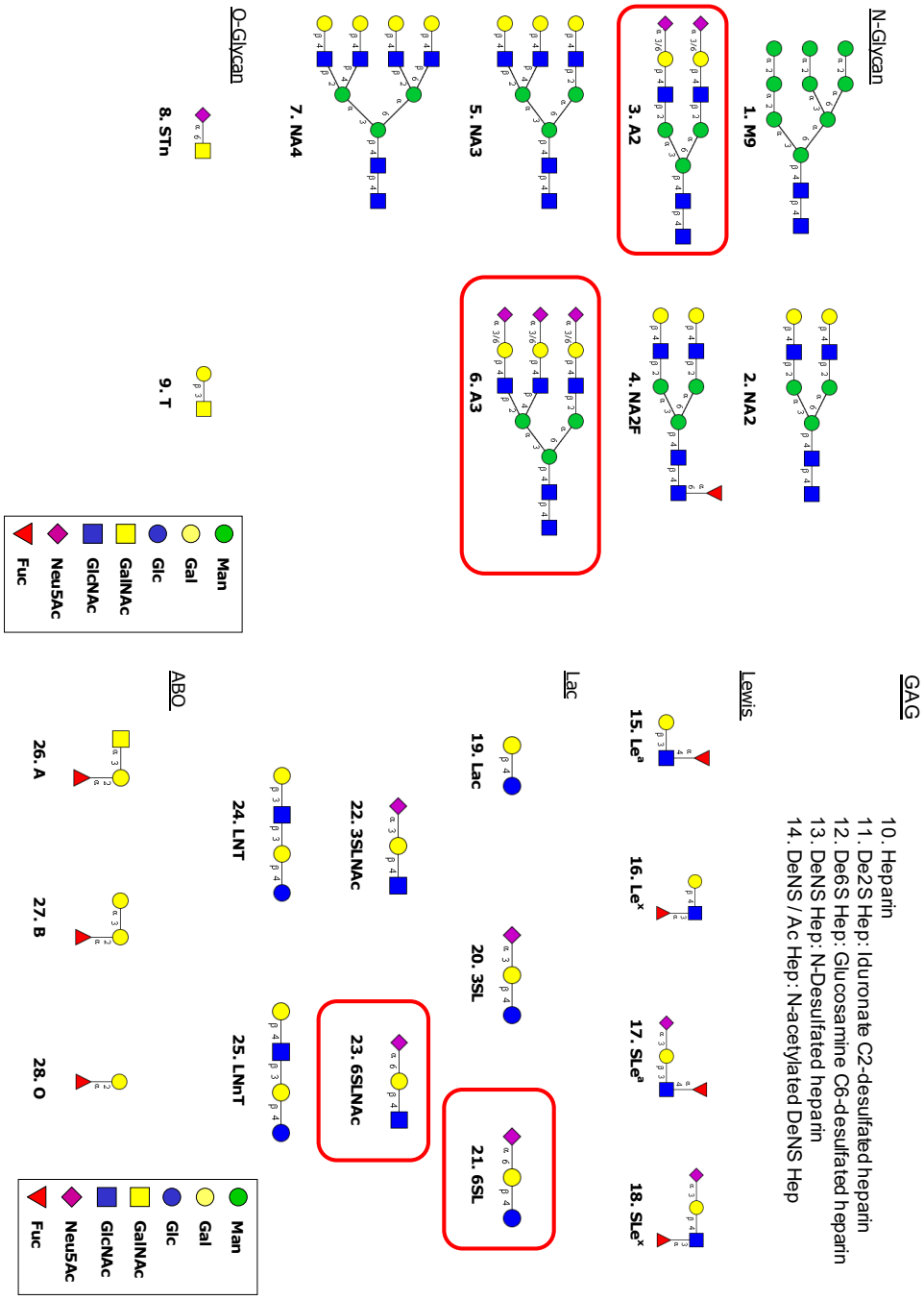
SI FIGURE 14-5.

Binding constants between 5-Bopi and SA (K_{SA}) for various pHs. K_{SA} is determined by plotting $[S_0]/P$ as a function of Q, where K_{SA} can be calculated by dividing K_{ARS} (obtained in SI Figure 10-2) by the slope of the plot equation. P and Q values were determined by equations from previous literature (Tetrahedron 58: 5291-5300 (2002)); S_0 total concentration of SA. Data are presented as means ± SD, (n=3).



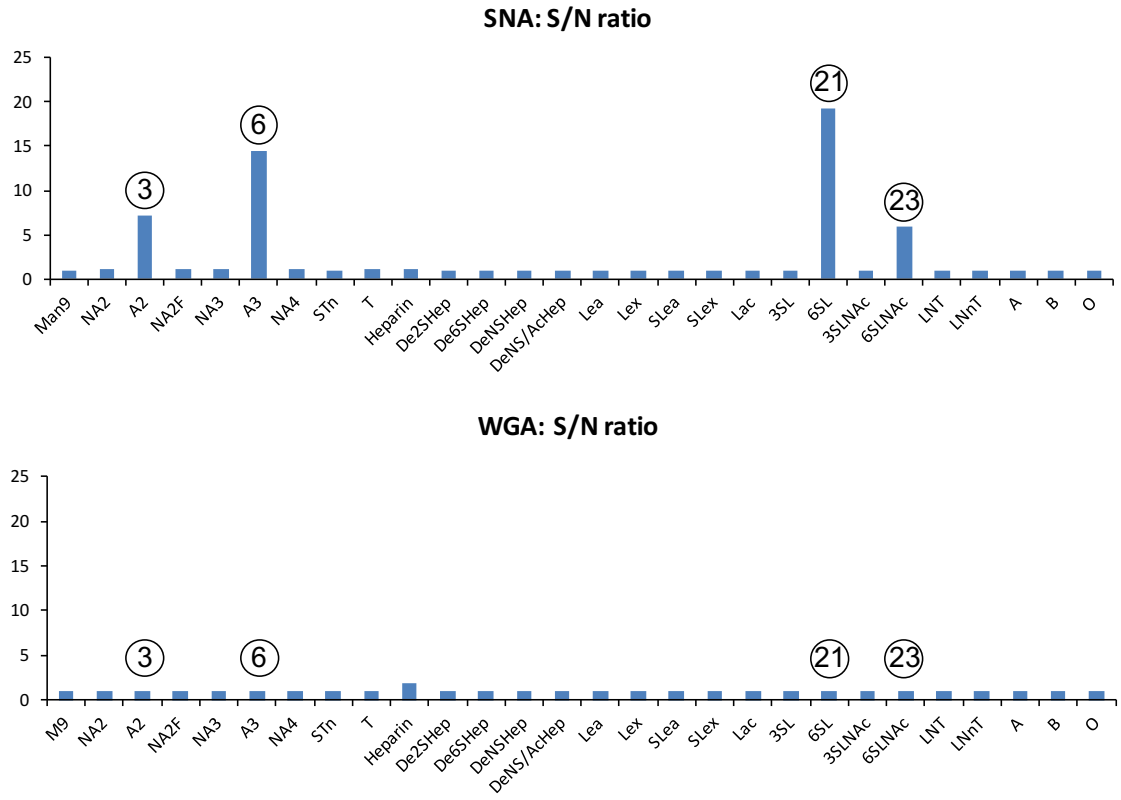
SI FIGURE 14-6.

Binding constants between 5-Bopi and SA (K_{SA}) as a function of pH, as determined by ARS assay (blue open circle) in comparison to those obtained from ^{11}B NMR analysis (black open circle: from Figure 1C). Data are presented as means ± SD, (n=3).



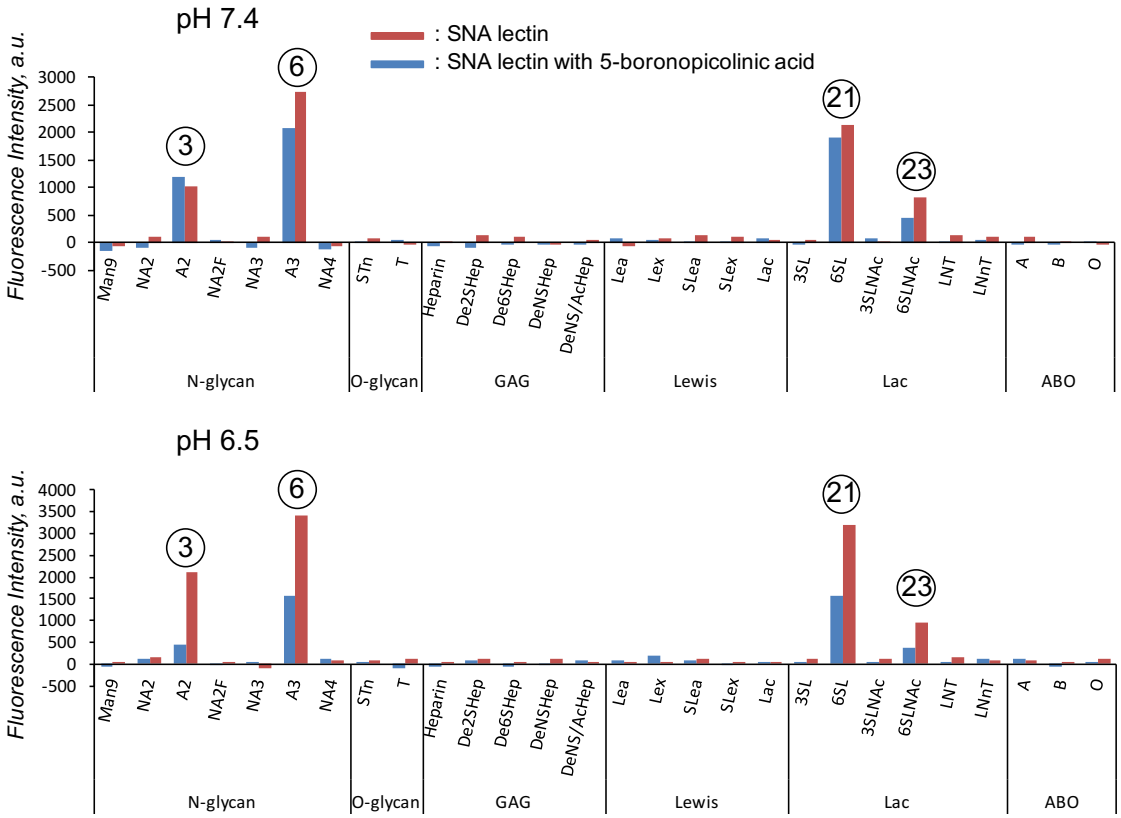
SI FIGURE 15.

Glycan types and structures installed on the array BS-X 1733Z (Type I: 28 glycans) (Sumitomo Bakelite Co., Ltd.); No. 3, 6, 21 and 22 (marked with red rectangles) are those with terminal Neu5Ac (α,6) GalNAc structures, to which SNA lectin has specificity.



SI FIGURE 16.

Glycan specificity assays for (*top*) SNA and (*bottom*) WGA lectins as assessed by glycan array (BS-X 1733Z: Sumitomo Bakelite Co., Ltd.).



SI FIGURE 17.

Competitive SA-binding assay between SNA lectin and 5-boronopicolinic acid for different pHs on the glycan array (BS-X 1733Z: Sumitomo Bakelite Co., Ltd.).

SI TABLE 1. Comparison of stability constants (at pH6) for SA binding to a series of boronic acid derivatives bearing different pKa, as determined by ^{11}B NMR.

Boronic Acid	Boronic Acid pKa	Stability Constants @ pH 6
5-Boronopicolinic acid	4.2	645
(6-Propylcarbamoyl)pyridine-3-)boronic acid	4.2	499
3-Pyridyl boronic acid	4.4	182
3-Borono-1-(carboxymethyl)pyridine	4.4	61
5-Pyrimidine boronic Acid	6.2	490
2-Fluoro-3-pyridyl boronic acid	6.3	419
Pyrazole-5-boronic acid	6.65	358
4-(2-acrylamidoethylcarbamoyl)-3-fluorophenylboronic acid	7.2	100
6-Methoxypyridin-3-boronic acid	7.99	34
3-Propionamidophenylboronic acid	8.3	40