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Molecular Recognition of Sialic Acid End Groups by Phenylboronates

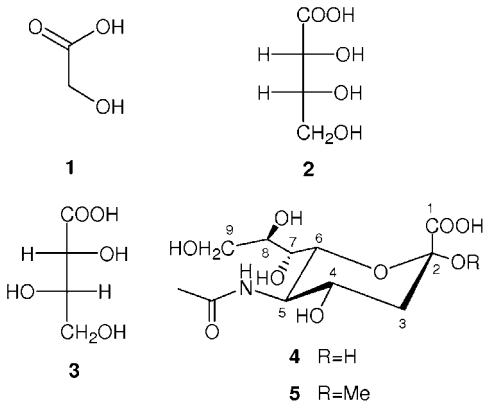
Kristina Djanashvili,[a] Luca Frullano,[a, b] and Joop A. Peters*[a]

Abstract: A multinuclear NMR study of the interaction between phenylboronic acid (PBA) and sialic acid (Neu5Ac) has been performed. The latter compound is known to be overexpressed on the cell surface of tumor cells. The results of this investigation suggest that the binding of PBA to sialic acid is pH dependent. ^{17}O NMR experiments with glycolic acid as the model compound prove that an interaction at the α-hydroxycarboxylate occurs at pH < 9, while a study with threonic and erythronic acids shows that the PBA group interacts selectively with the vicinal diol functions at higher pH. Similarly, Neu5Ac binds PBA through its α-hydroxycarboxylate at low pH (< 9) and through its glycerol side chain at higher pH values. The conditional stability constant of the phenylboronate ester at pH 7.4 is 11.4. On cell surfaces, sialic acid is connected to the neighboring sugar unit through the 2-hydroxy group. To mimic this the 2-α-*O*-methyl derivative of Neu5Ac was included in this study. The *erythro* configuration of the hydroxy substituents prevents stable-complex formation at positions C7 and C8 and, consequently, the strongest interaction is observed at positions C8 and C9, leading to a five-membered 2-boron-1,3-dioxalate. In addition, a relatively small amount of the C7–C9 six-membered complex was observed. Molecular modeling studies confirm that the C8–C9 boronate complex has the lowest energy.

Keywords: borates · carbohydrates · glycoproteins · molecular recognition · sialic acids

Introduction

The contrast in MRI images can be greatly improved by the use of contrast agents (CAs).[1] However, most of the currently available MRI CAs distribute rather unselectively over the extracellular space. If it were possible to accumulate CAs in the diseased tissue by means of molecular recognition, a much better contrast would be achievable. This has become feasible thanks to the increased knowledge of receptors, which allows the design of MRI CAs that respond to, for example, functional groups that are overexpressed due to disease.[2] Tumors are known to have cell surfaces on which sialic acids are overexpressed. Sialic acids contain the nine-carbon amino sugar neuraminic acid (Neu),[3] and the most common member of this family is 5-acetylneuraminic acid (**4**, Scheme 1).



Scheme 1. Structures of the polyols investigated in this study: (**1**) glycolic acid, (**2**) D-erythronic acid, (**3**) L-threonic acid, (**4**) 5-acetylneuraminic acid (Neu5Ac), (**5**) 2-α-*O*-methyl Neu5Ac.

[a] K. Djanashvili, Dr. L. Frullano, Dr. J. A. Peters
Laboratory for Applied Organic Chemistry and Catalysis
Delft University of Technology
Julianalaan 136, 2628 BL Delft (The Netherlands)
Fax: (+31) 15-278-4289
E-mail: j.a.peters@tnw.tudelft.nl

[b] Dr. L. Frullano
Chemistry Department, Northwestern University
Evanston, IL, 60208–3113 (USA)

Supporting information for this article is available on the WWW under http://www.chemeurj.org/ or from the author. 1) pH profile of the ^{11}B NMR chemical shift of PBA. 2) pH dependence of the ^{11}B NMR chemical shifts of a mixture of Neu5Ac and PBA. 3) ^{11}B NMR spectrum of a solution of Neu5Ac and H_3BO_3 at pH 11. 4) Energies and geometry of the calculated borate and PBA complexes of 2-α-*O*-methyl Neu5ac (Cartesian coordinates).

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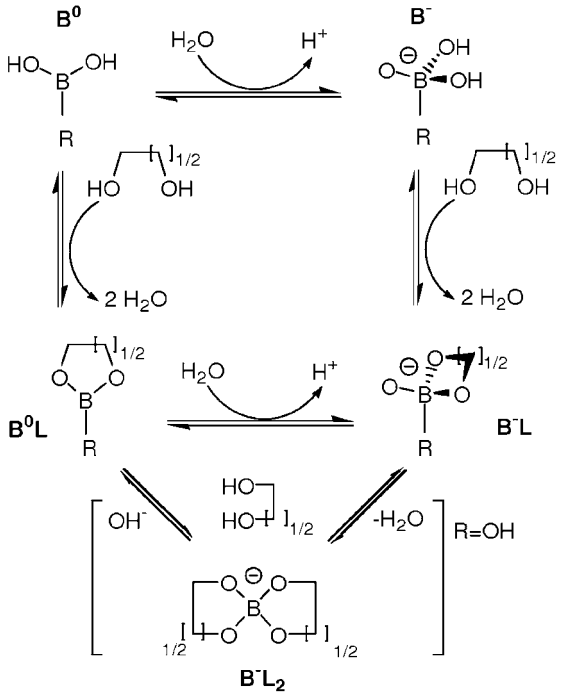
The importance of the sialic acid level as a diagnostic and prognostic indicator for different diseases has prompted the quest for specific targeting agents. Different approaches have been undertaken for this purpose. Bertozzi and coworkers have exploited the sialosides biosynthetic pathway to express a modified sialic acid, serving as an anchor for covalent binding of an aminoxy-substituted Gd-[DTPA]-complex.[4] The disadvantage of such a covalent targeting is that sialic acid catabolism occurs in lysosomes where the pH is relatively low (ca. 4–5). Under these conditions metal ions may dissociate from the chelate and exert toxic effects.

Currently, the only promising class of synthetic sugar receptors known is based on arylboronic acids. The ability of boric and boronic acids to form stable esters with polyhydroxy compounds is well known.[5,6] Boric acid can form cyclic esters with 1,2- or 1,3-diols in a 1:1 or 1:2 ratio; the possible equilibria between boric acid, borate, and the corresponding esters are summarized in Scheme 2.[7] The esters formed by borate anions, with the boron in its tetragonal hybridization, are always much more stable than the trigonal ones formed by boric acid. Phenylboronic acid behaves similarly to boric acid, although evidently it can only form esters with a 1:1 stoichiometry (Scheme 2). The presence of the aryl group also allows the design of more complex structures that can act as saccharide receptors.[8]



Scheme 2. Ester formation between 1,2- or 1,3-diols and boric acid (R = OH) or phenylboronic acid (R = Ph).

Some examples of receptors for sialic acid based on arylboronic acids have been reported in the literature. Shinkai et al. have developed a fluorescent sensor bearing a PBA moiety that is apt to interact with diols and the positively charged 1,10-phenanthroline–Zn^{2+} chelate moiety for carboxylate binding.[9] The two-site binding of sialic acid to the receptor was demonstrated by the fact that the association constant is much lower in the absence of Zn^{2+}.

Patterson et al. have explored carbohydrate receptors based on a poly(allylamine) (PAA) polymer substituted with both fluorescent arylboronic acid and carboxylic acid functions.[10] PAA with a 2% loading of the fluorescent arylboronic acid shows a response to sialic acid that is much higher than that for glucose, and slightly higher than that for fructose. Kataoka et al. have demonstrated that the PBA moiety in the copolymer of 3-(acrylamido)phenylboronic acid with *N,N*-dimethylacrylamide acts as an inhibitor for the cellular binding of an *N*-acetylneuraminic acid-specific lectin on lymphocytes.[11] The binding of PBA to the sialic acid moiety was studied by ^{11}B, ^{1}H, ^{13}C, and ^{15}N NMR spectroscopy with 3-(propionamido)phenylboronic acid as a model.[12] This compound shows an unusual pH dependence of the conditional stability constant of its esters with sialic acid, which was attributed to the formation of a trigonal boronic acid ester stabilized by an intramolecular B–N or B–O bond between the amide group of Neu5Ac and the boron atom.

The design of new targeting CAs capable of recognizing diseased tissue requires insight into the binding modes of PBA to sialic acid, which is overexpressed on the cell surface. Therefore we decided to study the interactions between PBA and the α-hydroxycarboxylic acids glycolic acid (**1**), erythronic acid (**2**), and threonic acid (**3**) as models for the various relevant functional groups in sialic acid (see Scheme 1). With the insight obtained it is possible to understand the binding of PBA to Neu5Ac (**4**) and its 2-α-methyl derivatives (**5**). The latter compound serves as a model for Neu5Ac bound to a neighboring sugar unit in glycoproteins.

Results and Discussion

Glycolic acid: Glycolic acid (HG) is the simplest α-hydroxycarboxylic acid and its interaction with PBA was studied to gain insight into the binding of PBA to the α-hydroxycarboxylic acid moiety in Neu5Ac (C(1)OOH and C(2)OH). The ^{11}B NMR spectrum of a mixture of glycolic acid (0.1 M) and PBA (0.1 M) displays a resonance for the equilibrium between free B^0 and B^- (Scheme 3). In addition, a second resonance at $\delta = -9.9$ ppm is observed between pH 3 and 9 (Figure 1). The chemical shift of this resonance is typical for a PBA ester, which has a tetragonal boron atom ($B^-G^{carboxylate}$, Scheme 3). The exchange between this species and B^0/B^- is slow on the ^{11}B NMR time scale. The concentration of $B^-G^{carboxylate}$ reaches a maximum at pH 6; at pH >8, its concentration is much lower, and at pH >10 it is no longer observable. The maximum in concentration of this species occurs when the pH value of the sample is between the pK_a of glycolic acid (4.21 ± 0.02) and that of PBA (10.05 ± 0.03) in the same solvent (see Supporting Information). This indicates that the ester concerned is formed by reaction of B^0

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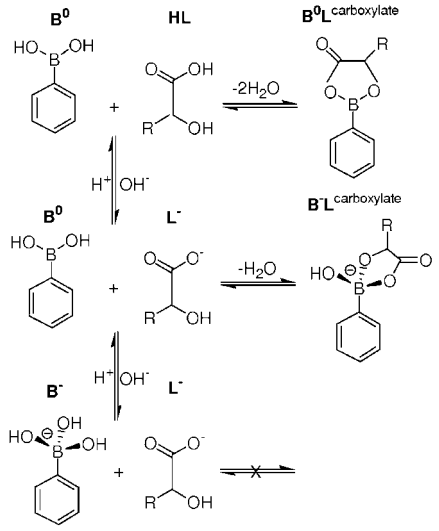

Scheme 3. Possible reactions between an α-hydroxyacid (glycolic acid R = H) and phenylboronic acid.

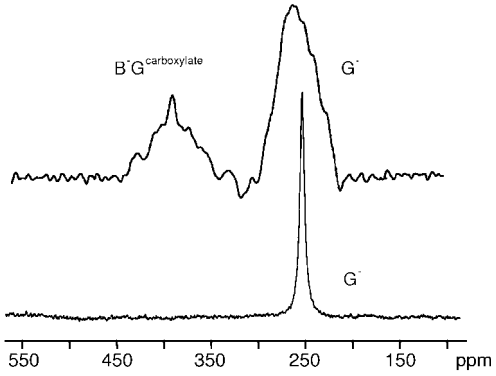

Figure 2. ^{17}O NMR spectrum of a solution of glycolic acid (0.8 M) and PBA (0.8 M) in methanol/water (1:2 v/v, 10% D_2O) at pH 7.3 (40.7 MHz, −15 °C).

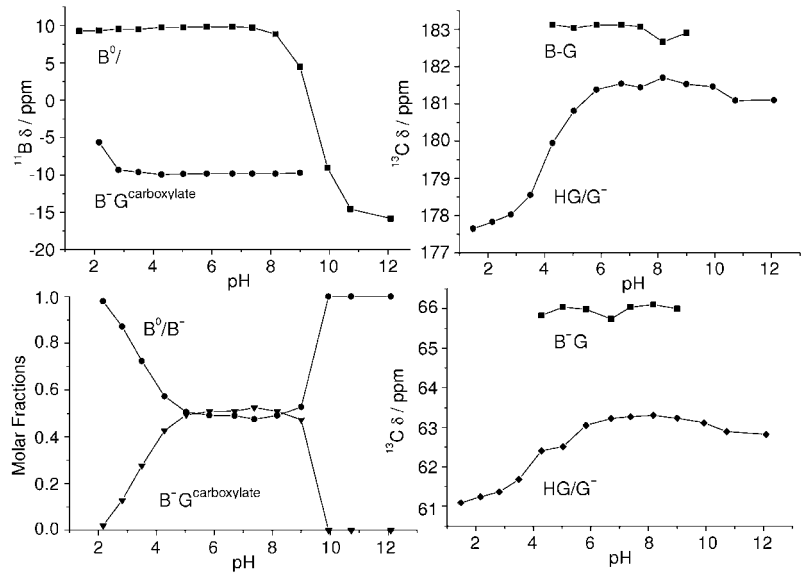

Figure 1. pH dependence of the ^{11}B NMR resonances (top left), molar fractions calculated from the integration of the ^{11}B NMR resonances (bottom left), and pH dependence of the ^{13}C NMR resonances in the carbonyl (top right) and aliphatic area (bottom right) for a mixture of glycolic acid (1 M) and phenylboronic acid (1 M).

with L^- (Scheme 3). A similar species has been observed in the glycolic acid–boric acid system.[7] The proposed binding mode of glycolic acid to the boron atom in the B^-L ester was supported by measuring the ^{17}O NMR spectrum of a sample in which the carboxylate group of glycolic acid was 10% enriched in ^{17}O. The spectrum of a 0.8 M solution of the ^{17}O-enriched glycolic acid in the absence of PBA at −15 °C shows a single relatively sharp resonance at $\delta = 253$ ppm. After the addition of one equivalent of PBA at pH 7.3 two broad resonances (at $\delta = 264$ and 391 ppm) became visible, signaling the loss of symmetry of the carboxyl group upon binding to boron (Figure 2). A similar behavior has been observed upon coordination of glycolic acid to Al^{3+}.[13] The signal at $\delta = 391$ ppm can be assigned to the carbonyl oxygen of the B-bound carboxylate. The other carbonyl oxygen atom is most likely coinciding with the resonance of the free carboxylate at about $\delta = 250$ ppm.[14]

The formation constant (K_f) of the B^-G ester, corresponding to the equilibrium shown in Equation (1), can be defined by Equation (3), whereas the acidity constant (K_{Gly}) of HG is given by Equation (2). The ^{13}C and ^{11}B chemical shifts for the species that are in rapid exchange on the NMR time scale can be described by Equations (6) and (7), respectively. Here, χ_n is the molar fraction of species n and δ_n is its intrinsic chemical shift. The molar fractions of free PBA ($B^0 + B^-$) and of the ester with glyconate (B^-G) were determined from the integrals in the ^{11}B NMR spectra.

$$B^0 + G^- \rightleftharpoons B^-G \quad (1)$$

$$K_{Gly=}[G^-][H^+]/[HG] \quad (2)$$

$$K_{f=}[B^-G]/[B^0][G^-] \quad (3)$$

$$C_{tB=}[B^0] + [B^-] + [B^-G] \quad (4)$$

$$C_{tGly=}[HG] + [G^-] + [B^-G] \quad (5)$$

$$\delta_i^C = \sum_n \chi_n^C \delta_n^C \quad (6)$$

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$$\delta_i^B = \sum_n \chi_n^B \delta_n^B \quad (7)$$

The value of K_f was evaluated to be 1.6 ± 0.2 by a simultaneous fitting of the ^{13}C and ^{11}B NMR shifts and the molar fractions of the boron species as obtained from the integrals, with Equations (2)–(7) To facilitate comparison of the various compounds discussed, we also defined a conditional stability constant at pH 7.4, K_f^c [see Equation (8)]. For glycolic acid K_f^c equals K_f.

$$K_f^c = [B^-G]/([B^0] + [B^-])([G^-] + [HG]) \quad (8)$$

Erythronic and threonic acid: Erythronic acid (HE) and threonic acid (HT) (see Scheme 1) were investigated because they contain both an α-hydroxycarboxylic acid and a 1,2,3-triol motif in their structures. Both moieties occur in Neu5Ac and are potential binding sites for PBA. It should be noted that the vicinal diol unit at C7/C8 in Neu5Ac has the *erythro* configuration. The ^{11}B NMR spectra of a mixture of equimolar amounts of erythronic or threonic acid (0.1 M) and PBA (0.1 M) as a function of pH are very similar for both sugar acids. Two signals are present in the entire pH range investigated, corresponding to free PBA (B^0/B^-) and to a convolution of the resonances of the various possible PBA–substrate esters (Figure 3). In both cases the chemical shift of the bound species, in the pH range up to 9, is about $\delta = -10$ ppm, which is similar to the value for the $B^-L^{carboxylate}$ species observed in the PBA–glycolate system. Therefore, it may be concluded that here also an α-hydroxycarboxylate ester ($B^-T^{carboxylate}$ or $B^-E^{carboxylate}$) with the boronate moiety bound to C1 and C2 is the predominant ester at pH 2–9.

Above pH 9, a shift of about 2 ppm towards lower frequencies is observed, indicating the disruption of the interaction at C1 and C2, which is consistent with the behavior of glycolate. However, now a new ester is formed that is in fast exchange with $B^-L^{carboxylate}$ (L = T or E) on the ^{11}B NMR time scale. In this high pH region, only interactions with the exocyclic triol moiety (at C2, C3, and C4) need to be considered. Three binding modes of B^- may be envisaged; 1) to C2 and C3, 2) to C3 and C4, and 3) to C2 and C4. The former two cases lead to five-membered boronate esters, whereas the latter gives a six-membered ring. It has been shown that five- and six-membered phenylboronate esters have chemical shifts of about $\delta = -12$ ppm and $\delta = -15$ ppm, respectively.[15] From the resonance at $\delta = -12$ ppm at pH > 10 in the present case, it may be concluded that binding to vicinal diol functions occurs to a considerable extent. The presence of six-membered esters cannot be excluded, however, because the resonance for this species would have a chemical shift of about $\delta = -15$ ppm and, therefore, may be obscured by the signal for free B^0/B^-. Between pH 2 and 9, the ^{13}C NMR spectra of 0.1 M erythronate or threonate in the presence of an equimolar amount of PBA are both similar to the spectra observed for the HG–PBA system (Figure 4). Two broad resonances are observed for the carboxylate group (C1), showing that for this nucleus the exchange between the boronate ester and the free acid is relatively slow on the NMR timescale. Broad and averaged signals are observed for all the other carbons, and only for the C2 carbon of threonic acid is a partial splitting visible. These spectra are consistent with the formation of a B^- $L^{carboxylate}$ ester at C1 and C2 under these conditions.

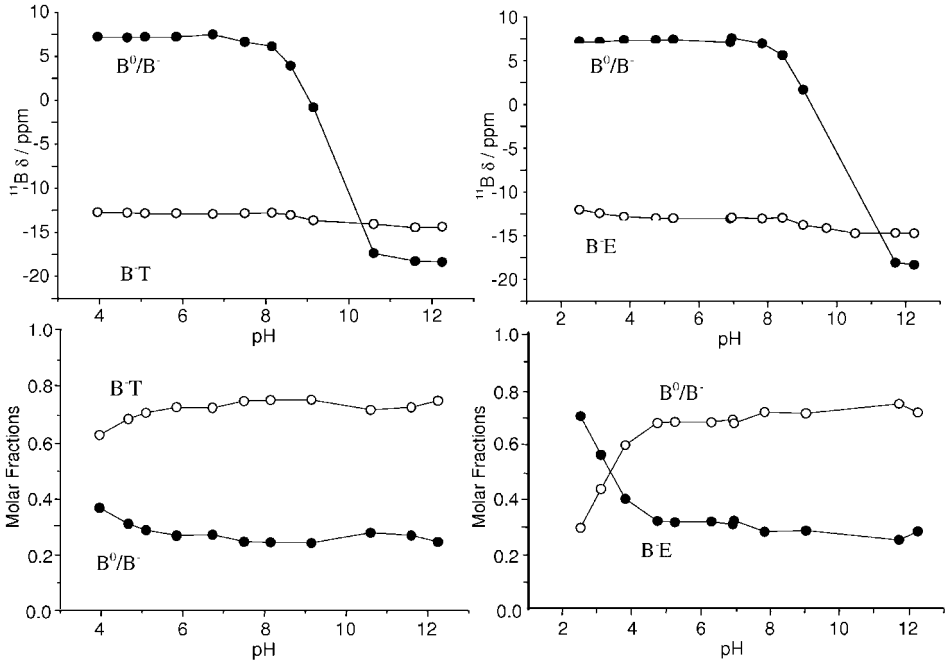

Figure 3. pH dependence of the ^{11}B NMR chemical shifts of mixtures of threonic (top left) or erythronic acid (top right) (about 1 M) and PBA (0.9 M) along with the molar fractions determined by the ^{11}B integrations (threonic acid: bottom left; erythronic acid: bottom right).

The spectra of erythronic and threonic acid in the presence of PBA become different above pH 9. In this region the spectra of erythronic acid display, besides resonances belonging to free PBA (B^-) and free erythronic acid (E^-), resonances corresponding to two pairs of B^-L^{diol} esters. Based on previous studies[7] on the interaction between H_3BO_3 and various poly(hydroxy)carboxylates, these resonances can be attributed to the pair of five-membered esters formed by interaction of PBA at C3 and C4. In

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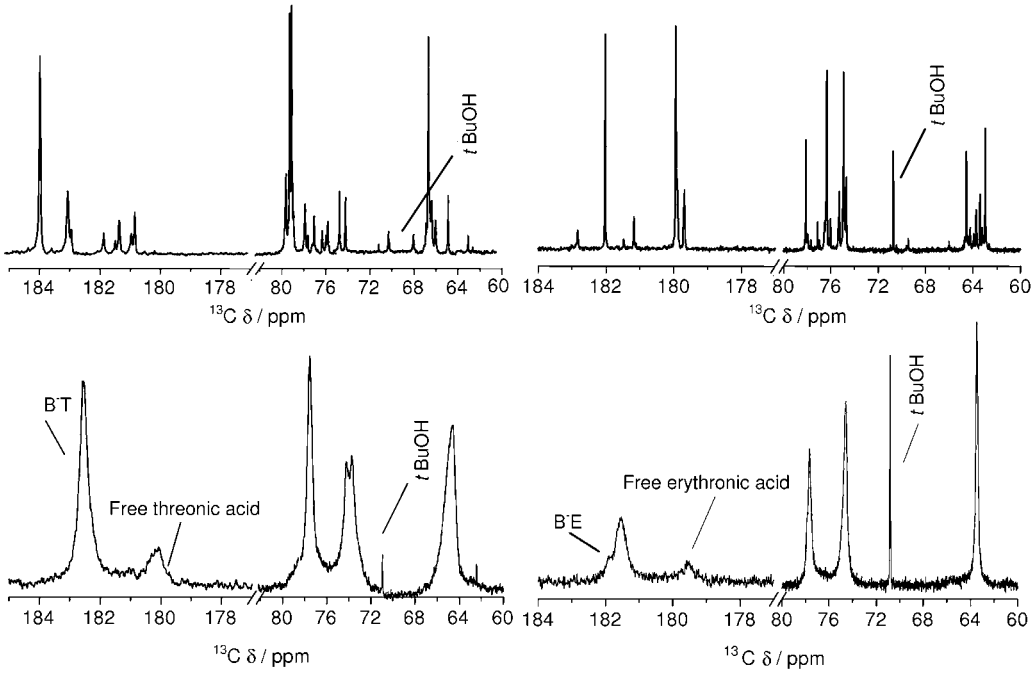

Figure 4. ^{13}C NMR spectra of a mixture of threonic acid (1 M) and phenylboronic acid (0.8 M) at pH 12.5 (top left) and 8.6 (bottom left), and of a mixture of erythronic acid (1 M) and phenylboronic acid (0.8 M) at pH 12.3 (top right) and 8.3 (bottom right).

these phenylboronate esters the B atom is chiral and, consequently, each of the interactions mentioned with the chiral α-hydroxycarboxylates results in two diastereoisomers. The major species observed at this pH can be assigned to the ester formed at C3 and C4. Formation of an ester at C2 and C3 can be excluded because of their unfavorable *erythro* configuration. Upon interaction at positions C2 and C3, the two terminal groups would assume a sterically unfavorable position and, moreover, the negatively charged boron atom would be relatively close to the carboxylate anion.

Besides these sets of signals, two sets of signals of relatively low intensity are visible, suggesting the presence of a small quantity of the six-membered ester originating from the interaction at positions C2 and C4 and of the five-membered ester formed at positions C2 and C3.

For the sample of threonic acid and PBA, besides the resonances belonging to free PBA (B^-) and free threonic acid (L^-), six sets of relatively sharp resonances are observed above pH 9. These resonances can be attributed to three pairs of diastereomeric B^-L^{diol} esters originating from the interactions at C2 and C3, C3 and C4, and C2 and C4. The major species is most likely one of the diastereoisomers of the ester formed with the diol at C2 and C3, which has a *threo* configuration.

The integrals of the ^{11}B resonances in the spectra of threonic or erythronic acid and PBA were used to calculate the conditional stability constant, K_f^c, at pH 7.4, as defined in Equation (8) for glycolic acid. For threonic acid we determined K_f^c to be $11.4 \pm 1.5\,M^{-1}$ and for erythronic acid $4.4 \pm 0.5\,M^{-1}$. The higher formation constant for threonic acid with respect to erythronic acid reflects the presence of a more favorable *threo* configuration at C2/C3 in the former compound.

The nature of the species observed at basic pH in the mixture of PBA and threonic acid (1:1) was confirmed by the study of the ^{11}B NMR spectra of a mixture of boric acid and threonic or erythronic acid (Figure 5). Borate and its esters

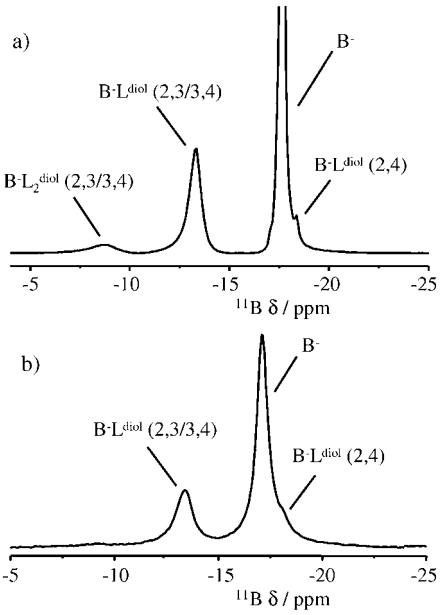

Figure 5. ^{11}B NMR spectra of a solution of threonic acid (1.0 M, spectrum a) and H_3BO_3 (2.2 M), and of a solution of erythronic acid (1.1 M, spectrum b) and H_3BO_3 (2.0 M) in methanol/water (1:2 v/v, 10% D_2O).

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have lower molecular volumes than the corresponding phenylboronate derivatives and, therefore, also lower rotational correlation times. Since the relaxation of ^{11}B is dominated by the quadrupolar mechanism, the ^{11}B relaxation rates of the boronate species are lower than those of the phenylboronate ones and, consequently, the linewidths of the ^{11}B NMR resonances of the borate esters are smaller than those of the corresponding PBA esters, which facilitates the identification of the different species. The ^{11}B NMR spectrum of the threonic acid–boric acid mixture at pH 11 shows four peaks at $\delta = -8.8$ (2.7%), -13.7 (24.6%), -17.7 (70.8%), and -18.4 ppm (1.9%) (Figure 5). From the chemical shift increments derived by Van Duin et al.,[7] these four resonances can be assigned to the B^-L_2 species ($\delta = -8.8$ ppm), the five-membered B^-L^{diol} species ($\delta = -13.7$ ppm, interaction at C2/C3 and C37C4), the six-membered B^-L^{diol} species ($\delta = -18.4$ ppm, interaction at C37C4), and to the free borate anion ($\delta = -17.7$ ppm).

The spectrum of the erythronic acid–boric acid mixture shows slightly broader resonances but the pattern is similar to that observed for threonic acid. Here also the resonance at $\delta = -13.4$ ppm (25.3%) is indicative of a five-membered B^-L^{diol} species due to interaction at C2 and C3 and/or C3 and C4, and the resonance at $\delta = -17.1$ ppm (54.8%) can be assigned to the free borate anion. The shoulder observed on this peak, at $\delta = -17.7$ ppm (19.2%), suggests the presence of a six-membered B^-L^{diol} ester. In this case the amount of B^-L_2 is negligible. A lower percentage of the bound species in the case of erythronic acid with respect to threonic acid is found in the form of five-membered esters, which reflects once again the presence of the unfavorable *erythro* configuration at C2/C3.

***N*-Acetylneuraminic acid**: From the results described above, it can be expected that *N*-acetylneuraminic acid (Neu5Ac) may interact with PBA through the α-hydroxyacid unit (C1/C2) and/or the glycerol moiety (C7–C9). The glycerol moiety of Neu5Ac has an *erythro* configuration between C7 and C8,[16] which is unfavorable for binding PBA.

The pH dependence of the ^{11}B NMR spectra of a solution of 0.6 M Neu5Ac in the presence of an equimolar amount of PBA is similar to that observed for erythronic or threonic acid. Two resonances were observed which could be assigned to the free PBA (B^-/B^0) and to a possible ester (B^-L), respectively. At pH < 8, the BL^- resonance is at about $\delta = -10$ ppm. The ^{11}B NMR chemical shift data of glycolic, threonic, and erythronic acid interacting with PBA have shown that this chemical shift is typical for a $B^-L^{carboxylate}$ species. Therefore, it can be concluded that, at pH < 8, the α-hydroxycarboxylate moiety at C1/C2 is involved in the binding of PBA. Around pH 9 this ^{11}B NMR resonance shifts to about $\delta = -12$ ppm, which shows that the PBA unit moves to the glycerol moiety at C7–C9 (see Supporting Information).

The integrals of the ^{11}B NMR resonances at pH 7.4 were used to calculate the conditional stability constant, K_f^c, defined similarly to Equation (8), for glycolic acid. The values of K_f^c obtained for Neu5Ac are compared with those for glycolic, threonic, and erythronic acid in Table 1.

Table 1. Conditional stability constants for the interaction between the investigated polyols and phenylboronic acid at pH 7.4 and 25 °C.

	K_f^c [M^{-1}]
glycolic acid	1.6 ± 0.2
erythronic acid	4.4 ± 0.5
threonic acid	11.4 ± 1.5
sialic acid	11.6 ± 1.9

The value of the conditional stability constant for Neu5Ac is much higher than that for erythronic acid under the same conditions. This may be rationalized by the increased distance between the boronate and the carboxylate anion, which results in lower charge repulsion between the two groups.

The importance of the α-hydroxycarboxylate moiety for the binding was confirmed by an investigation of the interaction of PBA with the 2-α-*O*-methyl derivative of *N*-acetylneuraminic acid (**5**). In this case the methyl group blocks the interaction of the boronic function at positions C1 and C2 leaving the glycerol tail as the only possible interaction site. As result, at pH < 8 no interaction is observable, while in the case of Neu5Ac an interaction with PBA is detected above pH 2 (Figure 6).

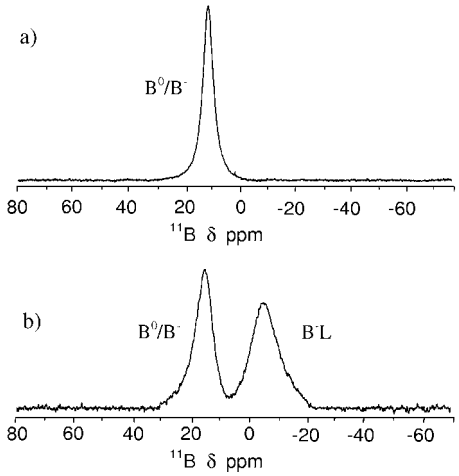

Figure 6. ^{11}B NMR spectra of a) α-methyl Neu5Ac (**5**) (0.05 M) and PBA (1:2.5) at pH 5.4, and b) Neu5Ac (0.6 M) and PBA (1:1) at pH 4.2 in methanol/water (1:2 v/v, 10% D_2O).

At pH values higher than 8 the binding to the glycerol tail of sialic acids becomes important; however, the broad ^{11}B resonances prevent the observation of separate peaks for the various esters formed upon interaction. The use of H_3BO_3, which gives sharper resonances, is therefore helpful for understanding the binding modes of the boronate function to **5**. At pH 11 the ^{11}B NMR spectrum of a solution of H_3BO_3 (instead of PBA) and **5** shows three resonances at

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$\delta = -13.9$ (14.3%), -16.8 (79.3%), and -18.1 (6.3%) ppm. The peak at $\delta = -16.8$ ppm can be assigned to free B^0/B^- (in fast exchange), whereas chemical shifts of $\delta = -13.9$ and -18.1 ppm are characteristic of five- and six-membered borate esters, respectively (see Supporting Information).

The ^{13}C NMR spectrum of **5** in the presence of an excess of PBA reveals, besides the signals for free **5**, three sets of resonances (two of which have relatively high intensity), thereby suggesting that at least two major and one minor phenylboronate esters occur in this solution (Figure 7). Un-

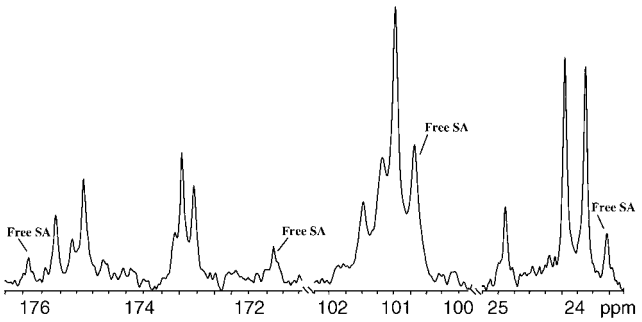

Figure 7. Fragments of the ^{13}C NMR spectrum of a mixture of α-methyl Neu5Ac (0.6 M) and PBA (1 M) at pH 11.5.

fortunately, the complexity of both ^{13}C and ^{1}H NMR spectra does not allow a complete assignment and evaluation of coupling constants, therefore these parameters cannot be used to discriminate between the various types of boronate esters. However, from the results of the ^{11}B NMR study on the borate esters (see above), it may be expected that both five- and six-membered esters are present in the sample with PBA as well. Since the B atom in these PBA esters is chiral, three pairs of diastereoisomers can be envisaged. An inspection of molecular models shows that some of these isomers have severe steric interactions. Semi-empirical calculations show that, in particular, the PBA esters at C7/C8 and C7/C9 with the B atom in the *S* configuration are highly strained. These calculations were performed without taking into account counterions or solvation effects, therefore the relative energies should only be considered as a crude measure of the stabilities. Similar calculations on the borate esters are qualitatively in agreement with the stabilities as determined by ^{11}B NMR spectroscopy. The results are compiled in Table 2.

Based on the results of these calculations, we conclude that the two major sets of peaks that are observed in the ^{13}C NMR spectrum can be assigned to the two diastereoisomeric five-membered esters with the boronate function attached to C8 and C9, whereas the minor compound is the *R* isomer of the six-membered ester at positions C7–C9.

The pattern of the interaction between PBA and Neu5Ac that emerges from our investigations differs substantially from that reported by Kataoka et al. for 3-(propionamido)-phenylboronic acid and Neu5A.[11] The occurrence of a relatively strong interaction at pH <7 can, in our view, be as-

Table 2. Relative energies of Neu5Ac-PBA/H_3BO_3 complexes obtained from the semiempirical calculations, compared to the complex with the lowest energy.

Complex	B-Binding site	ΔE [kJ]
Neu5Ac-borate complex	8–9	0
	7–9	10.8
	7–8	21.4
	8–9 *R*	0
	8–9 *S*	8.4
Neu5Ac-PBA complex	7–9 *R*	16.7
	7–9 *S*	35.2
	7–8 *R*	20.9
	7–8 *S*	33.5

cribed to an interaction with the α-hydroxycarboxylate moiety present at positions C1 and C2 of Neu5Ac. At pH >7, the glycerol tail of Neu5Ac becomes involved in the binding, and is the sole interaction site at pH >10. Kataoka et al. ascribed the high K_f^c observed at pH <7 to a stabilization of the trigonal boronic function bound at C7 and C8 of Neu5Ac. This interaction seemed to be supported by ^{15}N, ^{11}B, and ^{1}H NMR chemical shifts of the functions concerned and by the 2D ROESY NMR spectra of the Neu5Ac esters of another PBA derivative reported by Strongin et al.[17] An inspection of molecular models, however, suggests that both the B–N and B–O bonds would result in severe steric strain. For example, the separation of the B and the N atoms in this compound is significantly larger (3.5 Å) than the optimal length for a typical B–N bond, which is 1.6–1.7 Å.[18] The presence of a dative bond also does not agree with the ^{11}B NMR chemical shift, which was reported to be $\delta = -11.1$ ppm (pH 10), whereas a value of between $\delta = -4$ and -7 ppm would be expected for a B atom involved in a dative bond.[19]

Similar arguments hold for the ROESY data. The distances between the NH protons and various other protons in the PBA ester are strongly dependent on the rotations of the glycerol side-chain of Neu5Ac and cannot be directly related to the PBA derivative in question. All NMR spectroscopic data can equally well be interpreted by the structures described above for the PBA esters of Neu5Ac. The phenyl moiety in these compounds may give rise to substantial induced chemical shifts of the nuclei in its proximity. These shifts will be strongly dependent on the position of the phenyl group with respect to these nuclei.

The main binding sites of Neu5Ac for PBA are summarized schematically in Figure 8.

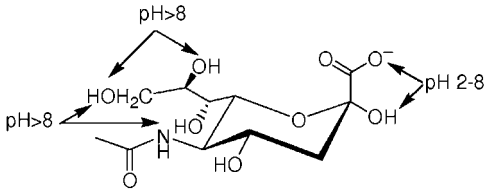

Figure 8. Schematic representation of the possible binding sites for PBA on Neu5Ac.

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Conclusion

From the results of the present study it can be concluded that phenylboronic acid forms esters with Neu5Ac between pH 2 and 12. At low pH (2–8), the α-hydroxycarboxylate moiety at C1/C2 is involved in the binding, whereas at pH >8 the binding of PBA takes place at the glycerol side chain to give a five-membered ester at C8 and C9. The formation of another five-membered complex at C7 and C8 is limited by the unfavorable *erythro* configuration of the glycerol tail. Molecular modeling studies confirm these conclusions, and show the lowest energies for the above-mentioned ester. The insight into the binding of PBA by Neu5Ac obtained in this study may be useful for the design of artificial receptors for sialic acid moieties in glycoproteins. On the cell surface sialic acids represent the terminal sugar residue of a glycan chain. That is, they are linked through C2 to positions 3 or 6 of the penultimate sugar or to position 8 of another sialic acid molecule.[20] Consequently, the carboxyl group is not available for interaction with the PBA receptor and, at physiological pH, it is the carrier of negative charge on the sugar molecule ($pK_a=2.2$). The experiment with the 2-α-*O*-methyl derivative of sialic acid demonstrates that interaction with PBA occurs even without carboxylic group participation.

More-selective and -effective artificial receptors for sialic acid residues in glycoproteins, therefore, should contain both a PBA unit and a group that is able to recognize the negatively charged COO^- group of sialic acid. Recently, we have successfully applied these principles in the design of a potential sialic acid targeting MRI contrast agents.[21]

Experimental Section

Compounds: D_2O was obtained from ARC Laboratories BV (Amsterdam, The Netherlands) and ^{17}O-enriched water (25% ^{17}O) from A. Matheson, USA Company (Miamisburg, Ohio). The water used for the preparation of samples was purified with a Milli-Q filtration system. Glycolic acid was purchased from Acros (Geel, Belgium) and *N*-acetylneuraminic acid (Neu5Ac) was purchased from Rose Scientific Ltd. (Edmonton, Canada). For some experiments Neu5Ac was converted into the methyl ester 2-α-*O*-methyl-5-acetylneuraminic acid according to a published procedure.[22] The structure was confirmed from the ^{13}C NMR spectrum (75.48 MHz, D_2O, 25°C, *t*BuOH, APT): $\delta=174.85$ (CO), 170.85 (CO), 100.38 (C), 72.27 (CH), 71.40 (CH), 70.19 (CH), 67.71 (CH), 65.30 (CH_2), 53.80 (CH), 53.17 (CH_3), 51.66 (CH_3), 41.58 (CH_2), 22.75 ppm (CH_3). During the ^{11}B NMR titration the ester group was hydrolyzed at high pH to form **5** in situ, as confirmed by the shift of the CH_3 peak at $\delta=53.17$ ppm in the ^{13}C NMR spectrum to $\delta=49.14$ ppm.

Threonic and erythronic acids were obtained from Aldrich as their calcium salts and were converted into the corresponding water-soluble sodium salts with a Dowex 50W×8 cation-exchange resin.

^{17}O-Enrichement of glycolic acid was achieved by stirring a 0.81 M aqueous solution of glycolic acid containing 4% of ^{17}O-labeled water at 80°C and pH ≈ 4 for a period of 16 h, followed by freeze-drying of the solution. ^{17}O NMR (40.67 MHz, D_2O): $\delta=253$ ppm (ref. H_2O, pH 5).

NMR spectroscopy: All NMR measurements were performed on Varian INOVA-300 and VXR-400S spectrometers at 25°C unless stated otherwise using 5-mm sample tubes. All NMR spectra were recorded using a water (10% D_2O)/methanol (2:1 v/v) mixture as the solvent. ^{13}C NMR spectra were measured at 75.48 MHz and *t*BuOH was used as an internal reference with the methyl peak of the standard set at $\delta=31.2$ ppm. ^{11}B NMR were measured at 128.33 or at 96.3 MHz, with a 0.1 M solution of boric acid in D_2O(δ=0.00 ppm) as external standard. The conversion to the $BF_3{\cdot}Et_2O$ scale is as follows: $\delta(H_3BO_3$ scale$)=\delta(BF_3{\cdot}Et_2O$ scale$)-18.7$. About 240 scans were collected using a delay and an acquisition time of 1 s. ^{17}O NMR spectra were recorded at 40.67 MHz. A spectral window of 29996 Hz and an acquisition delay of 0 s were applied. ^{17}O NMR spectra of mixtures of ^{17}O-enriched glycolic acid and PBA were acquired at −15°C to lower the exchange rates on the ^{17}O NMR time scale. The peak positions and the integrations of the resonances in ^{11}B, ^{17}O and quantitative ^{13}C spectra were determined by fitting the observed signals with Lorentzian line-functions.

Determination of stability constants: NMR-titration experiments were used to determine both the pK_a values of the studied compounds and the apparent stability constant (K_f) of the complexes. The pH values of the samples were measured at ambient temperature with a Corning 125 pH meter and a calibrated micro-combination probe purchased from Aldrich Chemical Company. The pH values of the solutions were adjusted with 1 M solutions of NaOH and HCl. The reported values are uncorrected for isotope effects and the presence of methanol. It has been reported that corrections for the solvent systems selected are negligible.[23]

Computer calculations were performed with the Micromath Scientist program, version 2.0 (Salt Lake City, Utah, U.S.A).

Molecular modeling: Calculations were performed with the HyperChem 7.5 Professional program. The MM+ force field was used to optimize the conformations of the possible complexes of Neu5Ac and PBA and to calculate the energies (given in $kJ\,mol^{-1}$). The conformations obtained were re-optimized by using AM1 semiempirical quantum mechanics. The default options (Restricted Hartree–Fock (RHF) spin pairing) were used with a total charge of −2 and a spin multiplicity of 1. Molecular dynamics at 1000 K and searches with the conformational search tool of the HyperChem software were performed to obtain the various conformational minima.

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