# MILD COLORIMETRIC DETECTION OF SIALIC ACID

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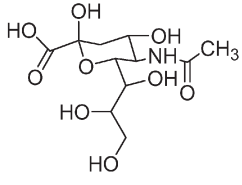
*Dedicated to Professor Ivan Stibor on the occasion of his 60th birthday in recognition of his outstanding contributions to supramolecular chemistry.*

As part of a broader program focusing on the detection of colorless biomolecules in the visible region, a simple and mild procedure for the visual detection of the most abundant sialic acid at room temperature and neutral pH is reported. The role of the interaction of the sialic acid amide with boron in a readily synthesized boronic acid-based receptor is an important feature of the signaling mechanism. Selectivity of reaction can be tuned by the judicious choice of solvents. This study embodies a departure from many of the author's earlier efforts in sugar solutions that are not heated, affording relatively more selective and milder detection.

**Keywords**: Sialic acids; Uronic acid; Sugars; Saccharides; Resorcinarenes; Calixarenes; Boronates; Boronic acids; Receptors.

Sialic acids are important cell surface residues. They occur in glycoproteins, glycopeptides and glycolipids. They play roles in cell to cell communication in humans and in the increased virulence in certain bacteria. Changes in sialic acid levels can lead to alterations in cell adhesion which may promote certain cancers as well as graft rejection. An increase in the levels of both soluble and cellular sialic acid can be a marker for cancer diagnosis[1].




**1**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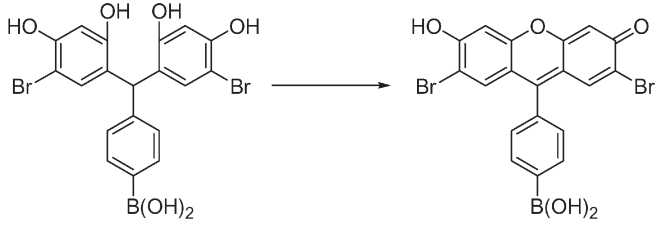
The most commonly occurring sialic acid is *N*-acetylneuraminic acid (NeuAc, **1**)[1]. The Warren and Svennerholm color tests are the most widely used in assays for sialic acid[2]. These methods are tedious and require high temperatures, harsh reagents and suffer from interference with other analytes[2]. In addition, many analyses require prior liberation of sialic acid residues from larger biomolecules to determine sialic acid levels. In the case of acid hydrolysis, destruction of sialic acids is observed[3]. During enzymatic hydrolysis incomplete sialic acid liberation can be a problem[2e].

The mechanism of signal transduction arising from the interaction of sugars with boronic acid-appended dyes is currently well-known[4]. We reported that calix[4]resorcarenes and structurally-related **2** form xanthene chromophores (e.g., **3**, Scheme 1) upon heating in DMSO solutions[5]. We demonstrated that heating aqueous DMSO solutions of **2** or boronic acid-derived resorcarene macrocycle-derived chromophores in the presence of sugars could be used for the detection of neutral and charged saccharides including **1** [5a]. Prior research in the area of boronic acid-promoted signaling of **1** has shown that there are difficulties in selective detection. For instance, uronic acids and fructose can be potentially significant interferents[6]. Herein we report new methodology towards a selective, mild and potentially non-hydrolytic assay for sialic acid. No heating of analytes solutions is necessary in the current study.

## EXPERIMENTAL

Compound **2** is synthesized according to our previously reported procedure[5b]. All other reagents and solvents are purchased from Sigma–Aldrich. HEPES buffer is prepared by dissolving HEPES in distilled $H_2O$ and adjusting the pH to 7.4 upon addition of NaOH. In order to




**2** **3**

SCHEME 1

The conversion of colorless **2** to chromophore **3**. The colored solutions are relatively stable for at least 36 h (≤1.4% absorbance change). Our previous studies[5c] have shown that ca. 1–10 μmol $l^{-1}$ concentrations of chromophores are typically produced in mmol $l^{-1}$ solutions of resorcinol/aldehyde condensation products such as **2**

promote chromophore (e.g., **3**) formation, stock solutions of **2** are pre-heated at a gentle reflux in DMSO for 3 min and allowed to cool to room temperature prior to all analytical studies. Sugar solutions are added at room temperature. Analyte-containing solutions are prepared in $H_2O$ (pH 7.4) prior to mixing with the DMSO/colored **2** solutions. No sugar-containing solutions are heated. All detection is performed at room temperature. The concentration of buffer in the $H_2O$ solution used in detection experiments is 0.26 mol $l^{-1}$. The final concentration of **2** used in all experiments is 5.2 mmol $l^{-1}$ (**3** is present at ca. μmol $l^{-1}$ levels[5c]). UV-VIS spectra are recorded on a Spectramax Plus (Molecular Devices). $^1H$ and $^{13}C$ NMR spectra are acquired in DMSO-$d_6$ on a Bruker DPX-250 or DPX-400 spectrometer. The concentrations of **1** and **2** in the NMR experiments are 100 mmol $l^{-1}$. All δ values are reported with DMSO at 2.49 ppm for $^1H$ NMR and 39.5 ppm for $^{13}C$ NMR as references. The 2D ROESY data are collected on a Bruker AMX-500 with a 5 mm $^1H/^{13}C$ dual probe, at 298 K, using a 9.2 μs pulse (90°). The delay between transients is 2 s, the mixing time is 300 ms, and 64 scans per increment are collected. A total of 2048 data points in F2 and 256 data points in F1 are collected over a spectral window of 12 ppm in both dimensions with an initial evolution time of 3 μs. Data are zero-filled to create a 2048 by 1024 matrix and processed using a squared sine (qsin) window function in both dimensions.

## RESULTS AND DISCUSSION

Colored aqueous DMSO solutions containing **2**, mixed with various analytes (in buffered $H_2O$, pH 7.4, DMSO–$H_2O$ 9:1) at room temperature, exhibit a selective color change for sialic acid (**1**) that is observable by visual inspection (Fig. 1). The analytes shown include known interferents in the Warren assay for sialic acid[2].

UV-VIS spectroscopy studies show that concentration changes of **1** can be monitored ratiometrically in the visible region (Fig. 2) in colored DMSO–

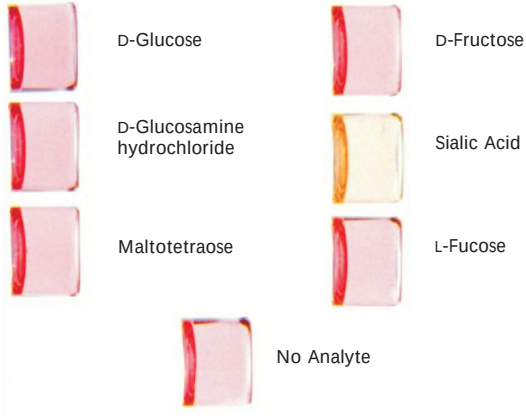



FIG. 1
Instant and selective color change observed upon addition of **1** to colored solutions containing **2** and **3**. Compound **3** is the active chromophore

$H_2O$ 9:1 solutions containing **2** (5.2 mmol $l^{-1}$). An increase in absorbance is observed at 475 and at 535 nm a concomitant decrease is observed upon increasing the concentration of **1**. Solutions of other carboxylic acid-containing sugars, such as glucuronic acid and galacturonic acid, exhibit weaker absorbance changes (535 nm) compared to **1** (Fig. 3). In addition, no observable spectroscopic or visible color changes are promoted by the addition of fructose, glucose, galactose, linear maltodextrins (maltose through maltoheptaose), glucosamine or acetic acid (at a concentration of

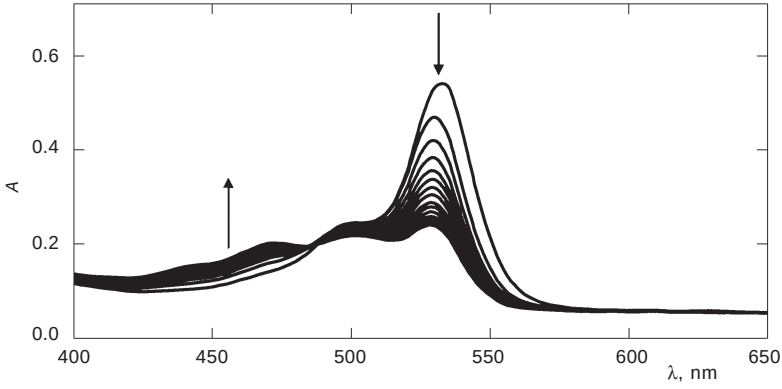



FIG. 2
Addition of **1** (0–14 equiv.) to colored solutions of **2** (5.2 mmol $l^{-1}$) and **3**. A blue shift is observed upon the initial addition of **1**

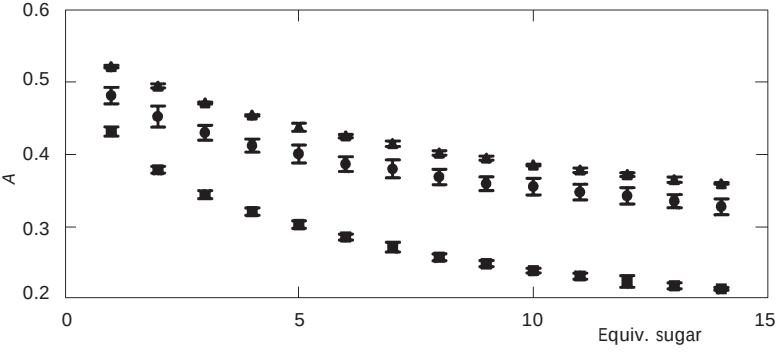



FIG. 3
Galacturonic acid (●), glucuronic acid (▲) and sialic acid (**1**; ■) each added to colored solutions of **2** (5.2 mmol $l^{-1}$) and **3**. The absorbance change at 535 nm is greatest for **1** compared to the other sugar acids. The contribution of the boron–amide interaction between **1** and **3** plays a role in binding and signaling. The error bars denote the range of absorbance values based on three sets of data

at least 45 mmol $l^{-1}$ of each) to colored chemosensor solutions. This indicates that this technique shows promise for the detection of **1** without interference from many commonly occurring neutral saccharides including oligosaccharides, aminosugars and certain carboxylic acid containing compounds.

Ratiometric absorbance increases at 475 (shorter visible wavelength $\lambda_{max}$) and decreases at 535 nm (longer visible wavelength $\lambda_{max}$) are well-known to occur in xanthene dye solutions upon raising solution acidity. Consistent with this behavior, we previously showed that an increase at 475 nm and decrease at 535 nm occurs upon increasing the acidity of solutions containing **3** [5b]. Based on pH titration experiments, we proved that the addition of sugars to solutions of **3** in buffered media also resulted in a significant absorbance decrease at 535 nm. The fact that this occurred in buffer solution showed that sugars lower the p$K_a$ of the chromophoric receptor. This is in keeping with the highly precedented behavior of boronic acid dyes upon binding to compounds containing *cis*-diols[4].

In addition, we demonstrated via NMR and IR spectroscopy, that acid-containing sugars can also lower the p$K_a$ of the chromophoric receptor **3** via charged hydrogen bonding to the xanthene oxygens in neutral media[5c]. Sialic acid (**1**), and glucuronic and galacturonic acid each contain carboxylate groups. In addition, galacturonic acid contains two *cis*-diol moieties and α-glucuronic acid contains one *cis*-diol. Thus, each of the three analytes promotes an absorbance decrease at 535 nm (Fig. 3).

Sialic acid, however, possesses acyclic triol functionality but no *cis*-diol configurations on the pyranose ring. Despite the fact that cyclic *cis*-diols are known to bind boronic acids much more effectively than their acyclic counterparts[4], we observed enhanced signaling for **1** as compared to the uronic acids. Boron–nitrogen interactions are well-precedented for lowering the working p$K_a$ of boronic acid dyes[4]. We thus attribute the relatively greater UV-VIS changes observed upon sialic acid binding to **3** to a boron–amide interaction, which cannot occur in the case of the uronic acids.

In contrast to **3**, most known boronic acid-functionalized dyes contain an aniline nitrogen which coordinates to a boronic acid moiety which is not conjugated to a chromophore or fluorophore[4]. This boron–nitrogen coordination effect is well-known to enhance the affinity of arylboronic acids for saccharides[4]. In the current study, we propose that an interaction between the amide of **1** and the boron atom of **3** promotes colorimetric selectivity over uronic acids and neutral sugars. The boron–amide interaction between **1** and 3-(propionamidophenyl)boronic acid has been recently demonstrated by NMR spectroscopy[7]. These prior studies showed that the

boron–amide interaction accounted for the known selectivity[8] of boronic acid for sialic acid (**1**) over glucose, mannose and galactose. Their evidence for the B–N/B–O interaction was demonstrated by $^{1}$H-$^{15}$N PFG-HMQC experiments. Significant chemical shifts for the amide NH proton and boron ($^{11}$B NMR) were observed. Studies involving sugars with greater affinity for boronic acids than **1** (e.g., fructose) or sensing were not contained within the scope of the previously reported work[7]. To the best of our knowledge, the study of a boron–amide interaction towards the selective colorimetric detection of sialic acid has not been previously reported.

$^{1}$H, $^{13}$C and 2D ROESY NMR results confirm the occurrence of the boron–amide interaction and the structures of **4** (in which the boron–amide interaction can occur) and **5** which are formed via the condensation reaction of **1** and **2** (Fig. 4). We observed an excellent correlation in chemical shifts with the analogous known complexes of (3-propionamidophenyl)boronic acid[7] and isomer **4** (Table I). Of particular note is the expected, characteristic upfield shift (from 8.06 ppm in solutions of uncomplexed **1**) of the amide nitrogen proton of **4** which appears at 7.69 ppm (literature value for the complex of **1** and (3-propionamidophenyl)boronic acid is 7.74 ppm [7]). In contrast, the amide nitrogen proton of complex **5** resonates at 8.10 ppm (literature value for the complex of **1** and (3-propionamidophenyl)boronic acid is 8.12 ppm [7]). The upfield shift for the NH proton of **4** is attributed to the interaction of the amide moiety with boron, which cannot occur in isomer **5** (Fig. 4).

In addition, an expansion of the key region of the 2D ROESY NMR (Fig. 5) of a mixture of **1** and **2** offers further evidence for an amide–boron interaction in complex **4**. The protons on carbons 4, 5 and 6 (see Fig. 4) exhibit through-space interactions with the NH proton of **4** while the pro-

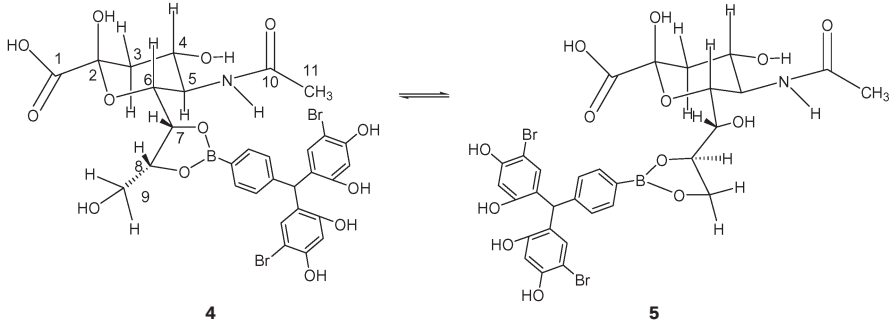



FIG. 4
Structures of complexes **4** and **5**

TABLE I
$^{1}H$ and $^{13}C$ NMR chemical shifts of complex **4**

| Atom[b] | $^{1}H$ and $^{13}C$ NMR chemical shifts, ppm[a] | |
|---|---|---|
| | (3-propionamidophenyl)-boronic acid[c] | experimental value compound **2** |
| H-4 | 3.80 | 3.80 m |
| H-5 | 3.86 | 3.89 d (8.71) |
| H-6 | 3.91 br d (10.3) | 3.97 br d (9.8) |
| H-7 | 3.97 br d (3.9) | –[d] |
| H-8 | 4.38 m | 4.30 m |
| N-H | 7.74 d (7.8) | 7.69 d (8.7) |
| C-4 | 66.63 | 66.60 |
| C-5 | 52.22 | 52.22 |
| C-6 | 70.22 | 70.23 |
| C-7 | 72.74 | 72.77 |
| C-8 | 65.44 | 65.63 |
| C-10 | 169.56 | 169.37 |

[a] Coupling constants (*J*) in Hz are shown in parenthesis (br, broad; d, doublet; m, multiplet). [b] Compound **4**, Fig. 4. [c] See lit.[7] [d] Overlapped.

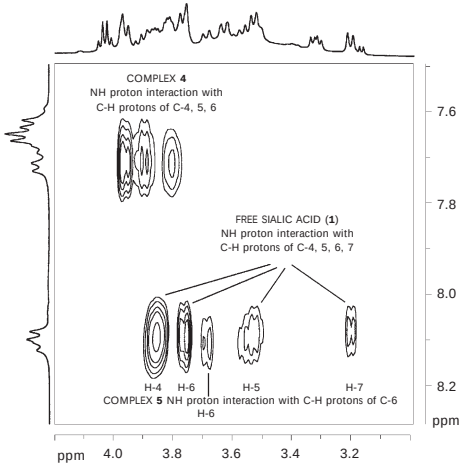



FIG. 5
Expansion of the 2D ROESY NMR of a mixture of **1** and **2** showing key interactions of **1** and **4**

ton on carbon 7 does not. An interaction between H-7 and the NH proton of compound **1** is, however, evident. We attribute the disappearance of the H-7/NH interaction in **4** to boronate ester formation involving the hydroxy groups on C-7 and C-8 of the glycerol moiety and concomitant boron–amide interaction. A significant interaction between the amide group and the boron atom in complex **4** would lead to restricted rotation of the glycerol side chain and necessarily force H-7 away from the amide NH. This study confirms prior NMR investigations of the boron–amide interaction[7] via a through-space, conformational analysis. It complements the prior studies[7] based on chemical shifts.

The interaction between boronic acids and diols is known to involve equilibria between neutral trigonal and anionic tetrahedral charge and hybridization states of boron[4]. The buildup of tetrahedral boronate anion, $ArB(OH)_3^-$ is relatively favored in $H_2O$ [4]. If the boron–amide interaction indeed plays a major role in the binding and signaling in a complex of **1** and **3**, then absorbance changes based on the interaction of **1** and **3** should diminish as the proportion of $H_2O$:DMSO is increased. This is due to the fact that $H_2O$, at increased concentrations, should effectively compete with

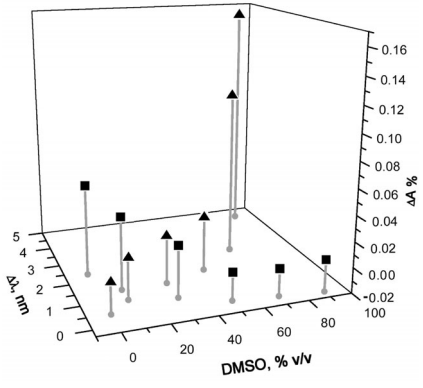



FIG. 6

The effect of solvent on the detection of sialic acid (**1**; ▲) or fructose (■) by **3** (concentration of **2** is 5.2 mmol $l^{-1}$). As the proportion of DMSO to $H_2O$ is increased, greater selectivity for **1** (2 equiv.) is observed via both a blue shift and absorbance decrease. As the proportion of $H_2O$ is increased, greater selectivity for fructose (2 equiv.) is observed via both a blue shift and absorbance increase. Compound **3** without added sugar exhibits a successive $\lambda_{max}$ blue shift from 535 to 500 nm upon changing the solvent proportion from 90% DMSO in $H_2O$ to 100% $H_2O$. The Δ-values displayed on the axes for $\lambda_{max}$ and absorbance refer to changes upon addition of fructose or glucose as compared to solutions of chromophore without sugar. The final concentration of HEPES buffer used in each of these experiments is 0.26 mol $l^{-1}$ (pH 7.4)

the amide of **1** for binding to the boron atom of **3** as a fourth ligand. Furthermore, selectivity towards sugars such as fructose, with known high affinities for boronic acids[8], should be enhanced upon increasing the proportion of $H_2O$ to DMSO, as the tetrahedral boronate anion, $ArB(OH)_3^-$, forms relatively strong covalent bonds to sugars in $H_2O$ [4]. In addition, cyclic *cis*-diols such as fructose preferentially bind to boronic acids which should afford selectivity for fructose over the acyclic glycerol moiety of **1**.

In support of this hypothesis, we found that the UV-VIS spectra of colored chemosensor **3** in the presence of **1** or fructose (both 10.4 mmol $l^{-1}$) change relative to variations in solvent proportions. As expected, upon increasing the proportion of $H_2O$:DMSO, selectivity towards fructose increases while sialic acid selectivity decreases. Figure 6 shows a plot of the changes in the $\lambda_{max}$ ($\Delta A$) shift vs the proportion of $H_2O$:DMSO in solutions containing **1** or fructose. Successively greater shifts in wavelength are observed as the % DMSO is raised in solutions containing **1**. The opposite effect is observed for fructose solutions; wavelength shifts successively increase as the proportion of $H_2O$ increases. In addition, we observed absorbance increases in solutions of fructose in 100% $H_2O$ ($\lambda_{max}$ 500 nm) by 5.1% (compared to a 0% increase in 10% $H_2O$ in DMSO, $\lambda_{max}$ 535 nm). Conversely, the absorbance change observed upon addition of **1** to colored solutions of **2** in 100% $H_2O$ (500 nm) is negligible. In 10% $H_2O$ in DMSO an absorbance decrease of 16.3% occurs (535 nm) in the presence of **1**. Judicious solvent choice thus plays a role in tuning the absorbance responses promoted by sialic acid, **1**.

## CONCLUSIONS

We have found that **1** can be detected in colored solutions containing **2** with excellent selectivity. The selectivity is attributed, in large part, to the precedented[7] coordination of the amide moiety of sialic acid to boron. We have confirmed the existence of the boron–amide interaction via 2D ROESY NMR. The moderate degree of signaling caused by uronic acids may be attributed to changes in the polarity of the microenvironment of the colored chemosensor or binding to the carboxylic acid[5c,9]. The investigation of these potential latter effects and their possible contribution to the sialic acid signaling observed is ongoing in our laboratory. The effect of solvent on selectivity has been investigated. The monitoring of sialic acid using our methods in media such as DMSO–$H_2O$ mixtures could lead to an efficient means of directly determining the sialic acid content of relatively hydro-

phobic important biomolecules such as gangliosides, which do not contain uronic acid moieties.

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