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Fluorescence and CD Spectroscopic Sugar Sensing by a Cyanine-appended Diboronic Acid Probe

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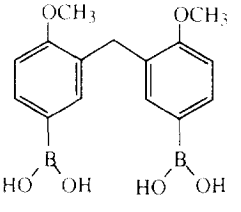
Abstract: A cyanine dye (**4**) bearing two boronic acids was designed and synthesized, expecting the selective binding of monosaccharides through the formation of 1:1 intramolecular complexes. While it aggregates in water, it exists discretely in water/methanol 1:1 mixed solvent. In the latter solvent the fluorescence spectra were scarcely affected by the medium pH but efficiently increased when it formed intramolecular 1:1 complexes with monosaccharides. This complexation mode was also corroborated by CD spectroscopy and continuous variation plots. Hence, the saccharide-induced fluorescence increase is rationalized in terms of "rigidification" of the cyanine skeleton. The association constants (K) were estimated from plots of saccharide concentration *vs.* fluorescence intensity: the largest K was observed for D-fructose (1.3×10^5 M^{-1}) and the next for D-arabinose (1.0×10^4 M^{-1}). This is a novel system for sensitive and selective fluorescence detection of monosaccharides.

Introduction

Molecular recognition of neutral and ionic species by synthetic receptors has been the facination of many chemists for the last few decades. In most reported synthetic receptors hydrogen-bonding interactions play a central role.[1] It is shown, however, that the hydrogen-bonding interactions are effective in aprotic solvents but less effective for recognition of guests soluble only in aqueous media. We are currently investigating the recognition of saccharides which are soluble only in aqueous media. Covalent-bond formation between saccharide and boronic acid has been utilized for sugar recognition in affinity chromatography by Wulff *et al.*[2] Recently, Shinkai and coworkers[3] reported the formation of rigid, cyclic complexes of diboronic acids **1** and **2** with mono- and di-saccharides. The induced chirality upon formation of rigid, chiral complexes was monitored by circular dichroism (CD) spectroscopy. Yoon and Czarnik[4] also reported the fluorescence suppression of anthrylboronic acid in the presence of saccharides. The suppression is due to the intramolecular fluorescence quenching by the boronate anion developed after complexation with saccharides.[4] In 1994, Sandanayake *et al.*[5] proposed a new class of artificial fluorescent receptors which respond to sugar substrates with two binding sites: *i.e.*, in *trans*-3,3'-stilbenediboronic acid **3**, the fluorescence intensity increases only when two boronic acids are intramolecularly bridged by di- or tri-saccharides.[5] The fluorescence increase was attributed to suppression of the rotational freedom in the ethylenic double bond, *i.e.*, to molecular rigidification. Although the fluorescence should be partially quenched by the boronate anions developed after complexation with saccharides as in anthrylboronic acid, the actual fluorescence increase suggests that the molecular rigidification

effect.

The research purpose of the present study is to extend the above-mentioned concept to fluorescence sensing of monosaccharides, the simplest but the most important family of saccharides. It is known that the selectivity is governed by the intramolecular distance between two boronic acids and therefore *trans*-3,3'-stilbenediboronic acid (**3**) favorably binds di- and trisaccharides.[5] We first attempted the synthesis of *trans*-2,3'- and 2,2'-stilbenediboronic acids which feature the shorter boronic acid-boronic acid distance but failed.[6] As an alternate idea, we decided to employ a cyanine dye (**4**) bearing two boronic acids. One can expect several advantages for **4**: for example, (i) although two boronic acids still keep some rotational freedom, the distance becomes comparable with the monosaccharide size when they adopt a *syn* conformation, (ii) compared with stilbene derivatives (in **3**, λ_{ex} and λ_{em} are 310 nm and 355 nm, respectively), both the excitation wavelength (λ_{ex} 480 nm in **4**) and the emission wavelength (λ_{em} 579 nm in **4**) in cyanine derivatives appear at longer wavelengths, which make the measurements easier, (iii) because of the presence of the multiple C=C double bonds between two aromatic skeletons, the greater rigidification effect is anticipated, which eventually leads to the greater fluorescence increase upon the monosaccharide binding, (iv) since the cyanine moiety and the phenylboronic acid moiety are separated by a CH_2 spacer, the boronate anions formed after complexation with monosaccharides scarcely quench the fluorescence: this situation also leads to the greater fluorescence increase, and (v) the synthesis is relatively facile. We have found that compound **4** thus molecular-designed and synthesized shows the markedly large fluorescence increase in the presence of monosaccharides and in addition, the absolute configuration can be conveniently predicted from the sign of the circular dichroism (CD) spectra.



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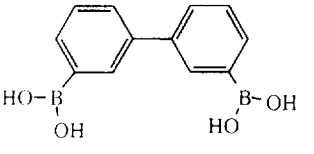

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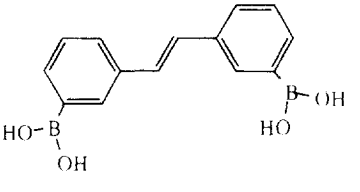

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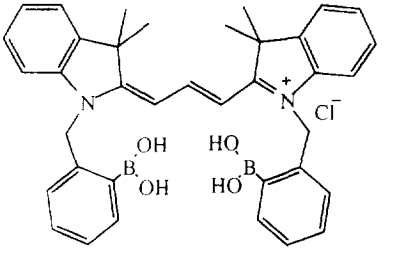


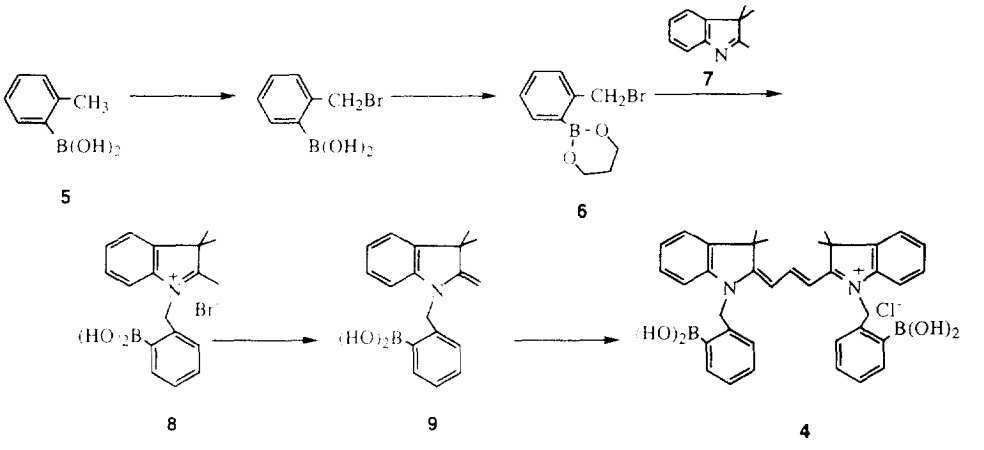
4

(in a *syn* conformation)

Experimental

Materials

Compound **4** was synthesized according to Scheme 1



Scheme 1

1,3-Dioxa-2-(2-bromomethylphenyl)borinane (6). This compound was synthesized after the method described in reference 7. *o*-Methylphenylboronic acid (**5** 5.0 g, 36.8 mmol), *N*-bromosuccinimide (7.86 g, 44.2 mmol), and AIBN (0.80 g, 4.86 mmol) were dissolved in anhydrous carbon tetrachloride (500 ml) and the solution was refluxed for 2 h under a nitrogen atmosphere. After cooling, the precipitate was removed by filtration and the filtrate was washed with water (200 ml). The organic layer was separated, dried over $MgSO_4$ and concentrated *in vacuo* to dryness. The reprecipitation of the residual solid from chloroform to hexane gave *o*-boromomethylphenylboronic acid in 55% yield. To identify the structure we converted this compound to **6**. *o*-Bromomethylphenylboronic acid (1.50 g, 6.98 mmol) was treated with 1,3-propanediol (0.76 ml, 10.5 mmol) in refluxing toluene for 2 h. After cooling, the solution was washed with water (50 ml), dried over $MgSO_4$ and concentrated *in vacuo* to dryness: slightly yellow oil, yield (from **5**) 52%. ^{1}H NMR (250 MHz, CD_3OD, 27 °C) δ 2.09 (-CH_2-C$\underline{H}_2$-CH_2-, m, 2H), 4.20 (-O-CH_2-, m, 4H), 4.91 (-CH_2Br, s, 2H), 7.19-7.78 (Ar-H, m, 4H) Anal. Calcd for $C_{10}H_{12}BBrO_2$: C, 47.12, H; 4.75 % Found: C, 47.34, H; 4.81%

3,3-Dimethyl-1-(2-boronylbenzyl)-2-indoline (9). This compound was synthesized after the method described in reference 8. To a boiling acetonitrile solution (40 ml) containing 2,3,3-trimethylindolenine (**7**: 2.38 g, 9.32 mmol) was added dropwise an acetonitrile solution (40 ml) containing **6** (1.35 g, 8.48 mmol). After 15 h the solution was cooled to room temperature and concentrated *in vacuo* to dryness. The residual reddish oil (**8**) was dispersed in water (100 ml) and water-insoluble unreacted **7** was extracted with diethyl ether. The aqueous solution was made alkaline (pH 10) with NaOH and stirred at room temperature for 1 h. The proton abstraction followed by prototropy affords compound **9**. The aqueous reaction mixture was extracted with diethyl ether, the organic layer being dried over $MgSO_4$. The solution was concentrated to dryness, the residue being purified by reprecipitation from chloroform to hexane: yellow powder, yield 48%, mp 172-174 °C; IR(KBr) ν_{OH} 3300 cm^{-1}, $\nu_{C=C}$ 1600 cm^{-1}, ν_{B-O} 1350 cm^{-1}. ^{1}H NMR (250 MHz, $CDCl_3$, 27 °C) δ 1.73 and 1.49

(-CH_3, s, 3H each), 4.55 (Ar-CH_2-, d, 2H), 6.14 (=CH_2, d, 2H), 6.84-7.93 (Ar-H, m, 10 H), **Anal.Calcd for** $C_{18}H_{20}BNO_2 \cdot 0.2C_6H_{14}$: C; 74.81, H; 6.93, N; 4.52 %. Found: C; 74.30, H; 7.41, N; 4.51%.

***N,N'*-Bis(2-boronylbenzyl)-3,3,3',3'-tetramethylindolinium chloride (4).** This compound was synthesized after the method described in reference 9. Compound **9** (50 mg, 0.51 mmol) and triethyl formate (0.17 ml, 1.02 mmol) were dissolved in pyridine (10 ml) and the solution was refluxed for 22 h. TLC examination (R_f = 0.48; silica gel, methanol/$CHCl_3$ = 1/10 (v/v)) at this stage showed that unreacted **9** still remains, so that we added triethyl formate (0.17 ml, 1.02 mmol) again and continued the reflux further for 5 h. After cooling, the solution was poured into an aqueous 1.0 M HCl solution and the mixture was stirred at room temperature for 30 min. The precipitate was recovered by filtration and purified by reprecipitation from chloroform to hexane: reddish power, yield 25%, mp (decomp.) > 238 °C; IR (KBr) ν_{OH} 3300 cm^{-1}, $\nu_{c=c}$ 1600 cm^{-1}, ν_{B-O} 1350 cm^{-1}; ^1H NMR (250 MHz, DMSO-d_6, 130 °C) δ 1.29-1.85 (-CH_3, m, 12H), 5.61 (Ar-CH_2-, s, 4H), 6.30 (C<u>H</u>-CH=C<u>H</u>-, d, 2H), 6.70-7.82 (Ar-H, m, 16H), 8.28 (CH-C<u>H</u>=CH, t, 1H). Anal. Calcd for $C_{37}H_{39}B_2ClN_2O_4 \cdot 1.5C_6H_{14}$: C; 72.49, H; 7.95, N; 3.68%. Found: C; 72.50, H; 7.94, N; 3.68%.

Miscellaneous

Absorption spectra, fluorescence spectra, CD spectra, and ^1H NMR spectra were measured with Shimadzu UV-160A, Hitachi 650-10S, JASCO J-720 and BRUKER AC-250P, respectively, unless otherwise stated. The buffers used to adjust the medium pH were 0.1 M acetate at pH < 5.8, 0.1 M phosphate at pH 5.8-8.0, 0.1 M carbonate at pH 8.0-11.0, and NaOH at pH > 11.0.

Results and Discussion

pH, Solvent, and Concentration Dependences. In general, cyanine dyes tend to aggregate in aqueous solution. We measured the absorption spectra of **4** in various water/methanol mixed solvents (Fig. 1). It is seen from Fig. 1 that the λ_{max} at 552 nm increases with increasing methanol concentration whereas it shifts to longer wavelength (λ_{max} 570 nm) with increasing water concentration. This result suggests that **4** (1.00 x 10^{-5}

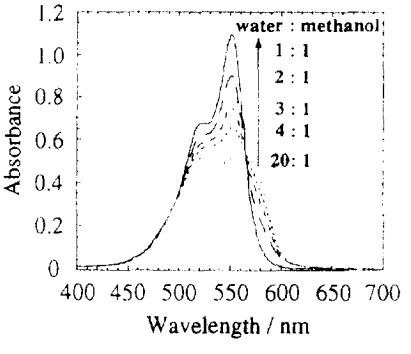


Fig. 1. Solvent dependence of the absorption spectra of **4** (1.00 x 10^{-5} M) at 25 °C. The solution was not buffered.

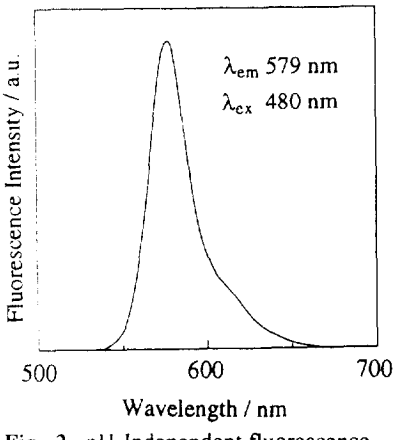
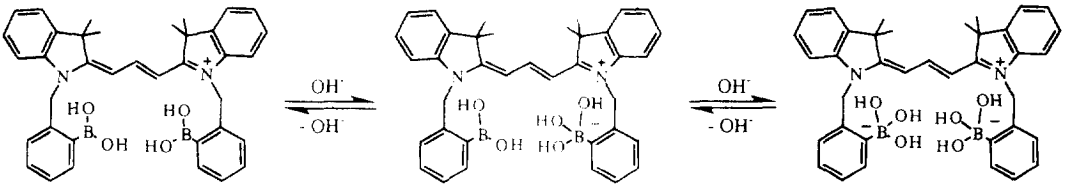


Fig. 2. pH-Independent fluorescence spectrum of **4** (1.00 x 10^{-5} M) in 1:1 water/methanol (v/v) at 25 °C.

M) aggregates in the mixed solvents containing water ≧ 10 vol%. On the other hand, one can regard that **4** exists discretely in 1:1 water/methanol (v/v) mixed solvent because (i) the spectral shape was scarcely changed by the further addition of methanol, (ii) a concentration-absorbance plot at (1-10) x 10^{-5} M obeyed the Lambert-Beer's law, giving a good linear relationship, and (iii) addition of sugars which should make **4** more hydrophilic and facilitates the deaggregation did not change the spectrum. We thus decided to use 1:1 water/methanol (v/v) as a standard solvent.

Compound **4** changes its structure in response to pH as shown in Scheme 2: it is monocationic at low pH region whereas it becomes monoanionic at high pH region. At intermediary pH region it is zwitterionic. However, as the pendent phenylboronic-acid groups do not conjugate with the cyanine dye skeleton, it is expected that their dissociation scarcely affects the absorption and fluorescence spectra at visible region.



Scheme 2

As expected, both the absorption spectra and the fluorescence spectra were independent upon the medium pH at pH 3~12. This situation makes pK_a determination by phototitration very difficult. To break this dilemma we decided to utilize the spectral change induced by the aggregation-deaggregation phenomena. As mentioned above, **4** changes its structure (from low to high pH) in the order of cationic → neutral (zwitterionic) → anionic species. This charge variation should be reflected by the aggregation properties, that is, by the spectroscopic properties. We thus measured the absorption spectra in 20:1 water/methanol (v/v) as a function of medium pH (the aqueous solution before mixing is buffered but the pH of the final mixed solvent is not corrected).

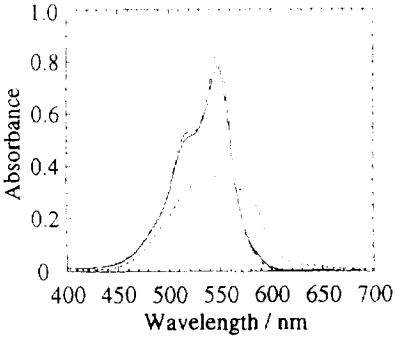


Fig. 3. Absorption spectra of **4** (1.00 x 10^{-5} M) at pH 2.32 (—), pH 8.03 (---), and pH 12.47(— -) in 20:1 water/methanol (v/v) at 25 °C.

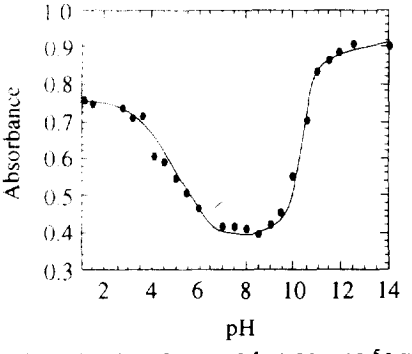
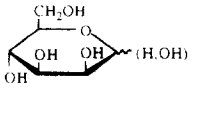


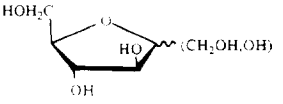
Fig. 4. Plot of A_{552} of **4** (1.00 x 10^{-5} M) *vs.* pH in 20:1 water/methanol (v/v) at 25 °C.

As shown in Fig. 3, **4** gave three different spectral shapes at acidic, neutral, and basic media. From a plot of the absorbance at 552 nm versus pH (Fig. 4) one can estimate the pK_a values to be 4.8 and 10.4. The large decrease in A_{552} at pH 5-10 is attributed to the aggregation in 20:1 water/methanol (v/v). Although these pK_a values should be somewhat different from those in 1:1 water/methanol (v/v), it is undoubted that the second pK_a exists at around 10. As seen in the related systems,[3-5] sugar complexation with boronic acid groups occurs with the aid of OH^- and lowers the pK_a by *ca.* 2 pK units. Hence, we chose pH 10.0 where boronic acids can form stable sugar complexes. We thus prepared a 1:1 water/methanol (v/v) solution from water (buffered to pH 10.0 with 0.1 M carbonate) and methanol and used it for subsequent spectroscopic measurements.

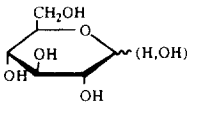
Fluorescence and CD Spectra. Here, we tested whether monosaccharides can intramolecularly bridge two boronic acids in **4** and rigidify the molecular structure. If they can, the fluorescence intensity would increase because of rigidification[5] and the complex would show the CD activity because of chiral orientation of the chromophoric cyanine group.[3-5,10,11] Here, we tested five monosaccharides; *i.e.*, D-mannose, D-fructose, D-arabinose, D-glucose, and D-xylose. It is already known that D-fructose shows the highest affinity with monoboronic acids among monosaccharides whereas D-glucose is frequently bound to diboronic acids selectively.[3-5,10-15]



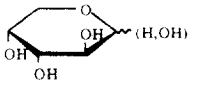
D-Mannose



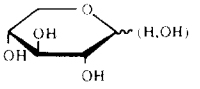
D-Fructose



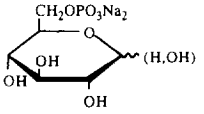
D-Glucose



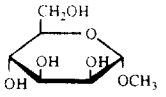
D-Arabinose



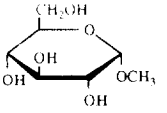
D-Xylose



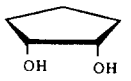
D-Glucose-6-phosphate disodium salt



α-Methyl-D-mannopyranoside



α-Methyl-D-glucopyranoside



cis-1,2-Cyclopentanediol

In Fig. 5 the relative fluorescence intensity is plotted against the monosaccharide concentration. The change is apparently biphasic consisting of a rapid fluorescence increase at low concentration region followed by a gradual fluorescence decrease at high concentration region. The similar biphasic dependence has been found for certain diboronic acids,[3,5,10,11] which is reasonably explained by the formation of an intramolecular 1:1 complex at low concentration region followed by the formation of a 2:1 saccharide/diboronic acid complex at high concentration region (Scheme 3).

In contrast, the fluorescence increase was scarcely detected for α-methyl-D-mannopyranoside, α-methyl-D-glucopyranoside, and D-glucose-6-phosphate disodium salt which can form only a 2:1 complex. Similarly, *cis*-1,2-cyclopentanediol which is known to form stable complexes with monoboronic acids[16] cannot induce the fluorescence increase.

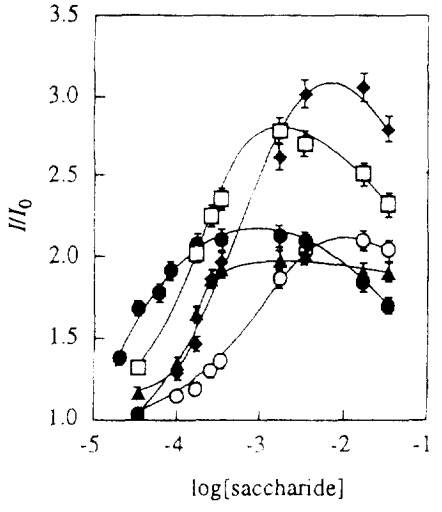
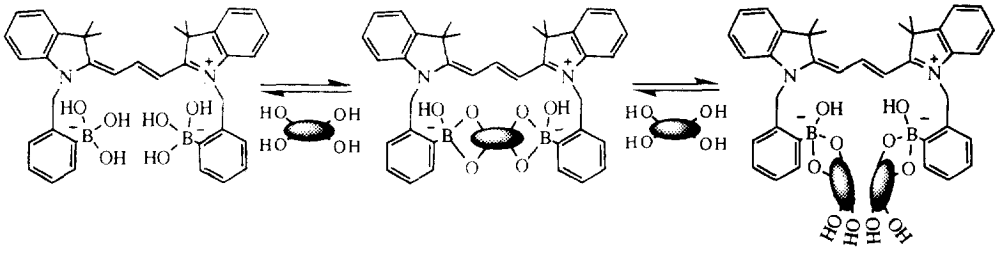


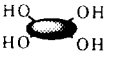
Fig. 5. Fluorescence intensity of **4** (1.00×10^{-5} M) plotted against monosaccharide concentration : water (pH 10.0 with 0.10 M carbonate)/methanol = 1:1 (v/v), excitation 480 nm, emission 579 nm, 25 °C (D-fructose (●), D-mannose (□), D-arabinose (▲),D-glucose (○), D-xylose (◆)).

These results consistently support the view that the fluorescence increase in the intramolecular 1:1 complex stems from "rigidification" of the cyanine skeleton by the saccharide bridge. Careful examination of Fig. 5 reveals that at high saccharide concentration region the fluorescence decrease is distinctly observable for D-mannose, D-fructose, and D-xylose whereas the gradual decrease is observable for D-arabinose and D-glucose. The macrocyclic 1:1 complexes in the former group are cleaved by the second saccharide attack to yield the 2:1 complex whereas those in the latter group are more stable and the shift from the 1:1 complex to the 2:1 complex occurs only at high saccharide concentration (Scheme 3). The results indicate that the 2:1 complexes are nonfluorescent (or at least much less fluorescent compared with the 1:1 complexes).



1:1 complex (fluorescent, CD-active)

2:1 complex (less fluorescent, CD-silent)


denotes a saccharide.

Scheme 3

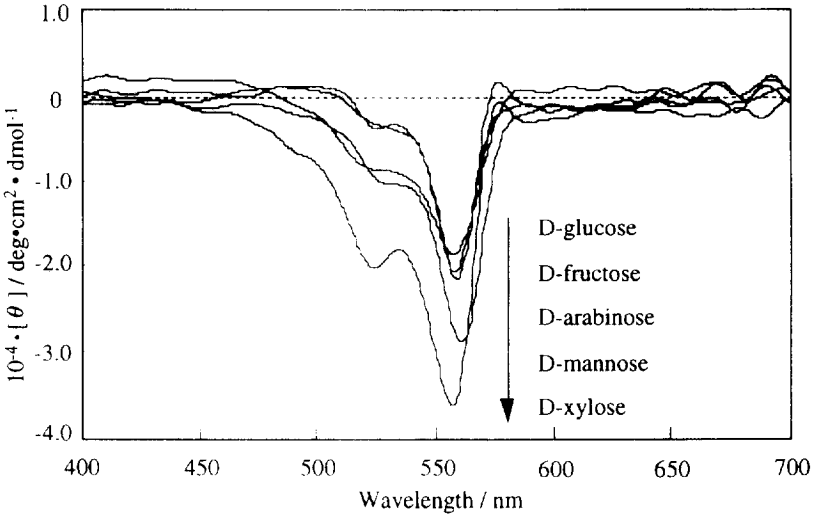


Fig. 6. CD spectra of **4** (1.00×10^{-5} M) in the presence of monosaccharides (1.67×10^{-3} M): water (pH 10.0 with 0.10 M carbonate)/methanol = 1:1 (v/v), 25 °C.

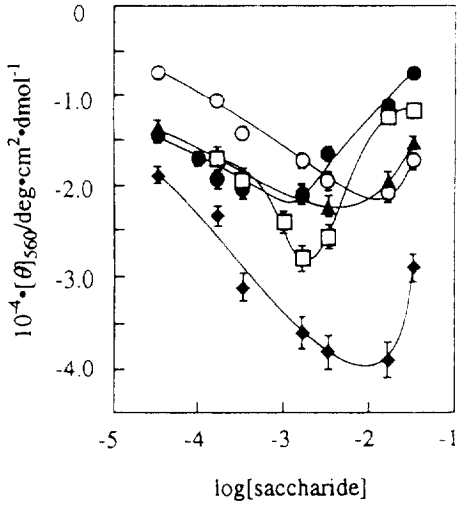


Fig. 7. $[\theta]_{560}$ of **4** plotted against monosaccharide concentration: water (pH 10.0 with 0.10 M carbonate) / methanol = 1:1 (v/v), 25 °C (D-fructose (●), D-mannose (□), D-arabinose (▲) ,D-glucose (○), D-xylose (◆)).

To obtain a further insight into chiral orientation of intramolecular 1:1 complexes we measured their CD spectra. As shown in Fig. 6, the saccharide-induced negative CD band was observed at 560 nm and the intensity at [saccharide] = 1.67×10^{-3} M was in the order of D-xylose > D-mannose > D-arabinose > D-fructose > D-glucose. To estimate whether the plots of $[\theta]_{560}$ *vs.* saccharide concentration are biphasic as in the plots of I/I_0 *vs.* saccharide concentration (Fig. 5), we measured $[\theta]_{560}$ for five saccharides as a function of log[saccharide] (Fig. 7). The biphasic dependence clearly seen for these saccharides reveals that the 1:1 complex formed at low concentration region is CD-active whereas the 2:1 complex formed at high concentration region is CD-silent (or at least much weaker than that of the 1:1 complex). To further confirm the 1:1 stoicheometry a continuous variation plot[17] was made (Fig. 8). The minimum at **[4]**/([**4**]+[D-fructose]) = 0.5 supports the view that the CD-activity stems from the formation of the 1:1 complex.

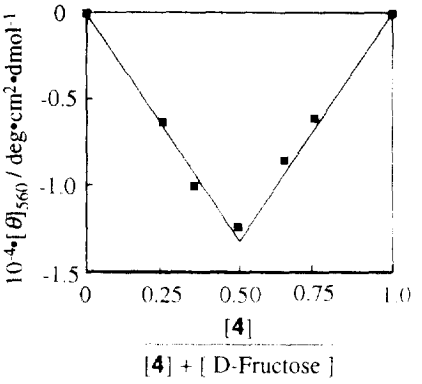


Fig. 8. Continuous variation plot for a **4** • D-fructose complex:water (pH 10.0 with 0.10 M carbonate)/methanol = 1:1 (v/v), 25 °C , [**4**] + [D-fructose] = 1.00 x 10^{-5} M).

By the analysis of I/I_0 *vs.* [saccharide] plots and $[\theta]_{560}$ *vs.* [saccharide] plots at low concentration region we determined the association constants (K) for the 1:1 complex. The results are summarized in Table 1. It is seen from Table 1 that the K values determined from the fluorescence spectrta show good agreement with those determined from the CD spectra. The largest K value is obtained for D-fructose and the next for D-arabinose. On the other hand, the K value for D-glucose is relatively small although it is still much greater than those with monoboronic acids.[4,12-15] Conceivably, being different from precedent diboronic acids which show D-glucose selectivity,[3,10,11] two boronic acids in **4** are not arranged as to cooperatively capture D-glucose as a 1:1 complex. We also tried to determine the association constants for the 2:1 complexes by a nonliner least-squares method. However, the concentration range useful for the computation is so narrow that we could not obtain the reliable data.

Table 1. Association constants [a]

Saccharide	K / M^{-1} (from I/I_0)	K / M^{-1} (from $[\theta]_{560}$)
D-Fructose	1.3 x 10^5	1.6 x 10^5
D-Mannose	5.9 x 10^3	5.6 x 10^3
D-Arabinose	1.0 x 10^4	8.4 x 10^3
D-Glucose	1.4 x 10^3	2.3 x 10^3
D-Xylose	4.8 x 10^3	6.6 x 10^3

[a] The association constants were determined by using Benesi-Hildebrand equation.[18]

Conclusions

Thus, the present paper has demonstrated that compound **4**, a diboronic-acid-appended cyanine dye acts as a useful fluorescent sensor for monosaccharides. The essential points realized in the present molecular design are to rigidify the cyanine skeleton by macrocyclization and to adjust the distance between two boronic acids to the size of monosaccharides. We believe that the concept should be applicable more generally to the design of fluorescent sugar sensors.

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