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Synthesis, Evaluation, and Computational Studies of Naphthalimide-Based Long-Wavelength Fluorescent Boronic Acid Reporters

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Abstract: Boronic acids that change fluorescence properties upon sugar binding are very useful for the synthesis of carbohydrate sensors. Along this line, boronic acids that fluoresce beyond 500 nm are especially useful. A series of boronic acid fluorescent reporter compounds based on the 4-amino-1,8-naphthalimide structure have been synthesized (**1a–d**) and evaluated under near physiological conditions. These compounds showed good water solubility and significant changes in fluorescence properties after binding with sugars, with the emission wavelength being at around 570 nm. Analogues in this series with different substitutions showed similar properties. We have also examined the mechanism of the observed fluorescence changes for these compounds.

Keywords: boronic acids · carbohydrates · fluorescence · sensors

Introduction

Boronic acids are known to bind 1,2- and 1,3-diol-containing structures through reversible covalent interactions.[1–4] Such interactions have been used for the preparation of fluorescent sensors[5–23] and transporters[24,25] for carbohydrates, as well as lectin mimics (termed boronolectins) for cell-surface carbohydrate recognition.[26–30] Critical to such sugar-sensing efforts is the availability of boronic acid reporter compounds that change fluorescence properties upon sugar binding. Particularly desirable are boronic acids that emit fluorescence at long wavelengths beyond the UV region (above 500 nm), which would have less background interference and higher penetrating power for cellular applications.

Some long-wavelength fluorescent boronic acid reporters have been reported.[7,9,15,31–35] Among those compounds, a boron–dipyrromethane (BODIPY) dye functionalized with a phenylboronic acid group (510 nm) was the only water-soluble fluorescent probe that showed fluorescence intensity increases upon sugar binding.[36] Long-wavelength emission has been observed with the 1,8-naphthalimide fluorophore, and the addition of a 4-amino group to this compound has been shown to significantly change its fluorescence properties.[12,37–39] In our recent work, 4-amino-1,8-naphthalimide was used as a template for a series of long-wavelength fluorescent boronic acid reporters that showed fluorescence intensity increases upon sugar binding.[13] Somewhat related work was also reported by Trupp and co-workers. However, in their work, an added aliphatic linker with an appended amino group was used to modulate the fluorescence properties of a naphthalimide fluorophore.[12] In our work, the phenylboronic acids are directly attached to the naphthalimide fluorophore to modulate its fluorescence. In this series, an amino group was positioned in a 1,5 relationship to the boronic acid, which helps to modulate its pK_a and electronic states.[6,40–43] The change in the ionization state of the boron atom upon sugar binding from the neutral sp^2 form to the anionic sp^3 state was thought to be the reason for the observed fluorescence changes. In our previous work, we examined the effect of N substitution on the fluorescence properties of this series of compounds. Herein, we would like to report our work on the design, synthesis, and evaluation of **1b–d**, compounds that have electron-donating or electron-withdrawing groups at the *para* position on the phenyl boronic acid moiety (Scheme 1). These groups include a methoxy group (**1b**), a methoxycarbonyl group (**1c**), and a fluoro group (**1d**). We were interested in studying whether changes in the phenylboronic acid substitution would have a significant effect on the fluorescence and binding properties of these compounds. Moreover, we also used computational chemistry to study the theoretical basis for

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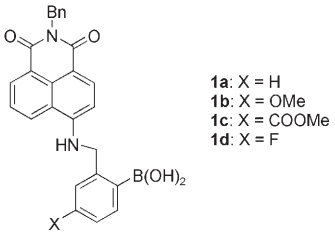
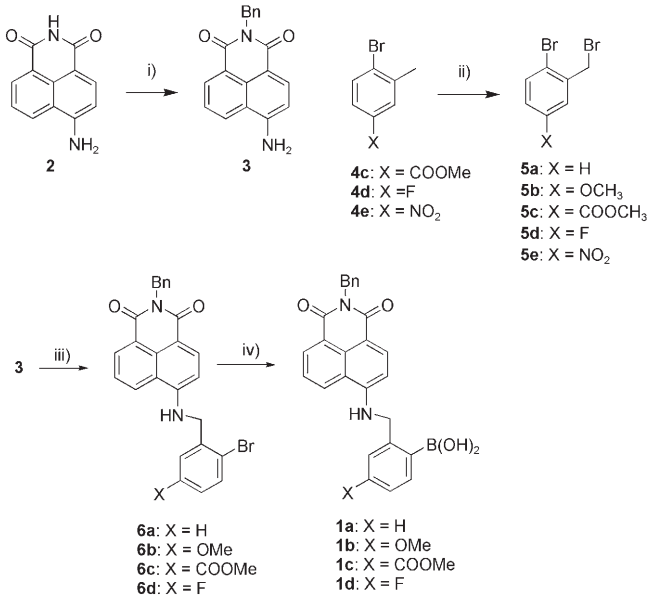


Scheme 1. Structures of long-wavelength boronic acid reporters. Bn: benzyl.

the observed spectroscopic property changes. The compounds synthesized will be very useful for the preparation of fluorescent carbohydrate sensors and lectin mimics (boronolectins),[29] and the understanding of the substituent effects gained will also be very useful for the future design of other fluorescent boronic acid compounds.[29]

Results and Discussion

Synthesis: For the synthesis of the analogues designed, our general approach was to couple the 4-amino-1,8-naphthalimide core (**2**) with an appropriately substituted 2-bromobenzylbromide (**5a–e**, Scheme 2). For this, we needed to protect the imide nitrogen atom of **2** to minimize side reactions. A benzyl protecting group was used. In our previous work, we also studied other protecting groups and found that use of the methoxymethyl (MOM) protecting group resulted in chelation of the palladium catalyst in the borylation step and subsequently diminished borylation efficiency.[13] Therefore, in the first step of protection, 4-amino-1,8-naphthalimide (**2**) was treated with sodium methoxide/methanol in DMF at room temperature and was then treated with benzyl bromide to afford 4-amino-*N*-benzyl-naphthalimide **3** (Scheme 2). Among the alkylating agents **5a–e**, three were not commercially available and were prepared through benzylic bromination of the corresponding bromotoluene analogues **4c–e** by treatment with AIBN and NBS in CCl_4 at 70 °C (reflux conditions). Subsequent alkylation of **3** with benzylbromide analogues **5** gave the desired arylbromide compounds **6** except with **5e**, which did not undergo alkylation. Borylation of **6a** was carried out with $PdCl_2$(dppf) as the catalyst to generate **1a**.[44–46] Boronic acids **1b–d** were prepared from **6b–d** by following similar procedures. It should be noted that deprotection of the boronic acids occurred spontaneously during the borylation reaction and the subsequent workup. Therefore, no separate deprotection step was needed. Purification of the final products by using C-18 reversed-phase (RP) HPLC afforded free boronic acids **1a** (30%), **1b** (24%), **1c** (14%), and **1d** (21%).



Scheme 2. a) $NaOCH_3/CH_3OH$, BnBr, DMF, room temperature; b) NBS, AIBN, CCl_4, reflux, $h\nu$; c) NaH, DMF, **5a–d**, room temperature; 4) neopentylglycol diboron, [$PdCl_2$(dppf)], KOAc, DMSO, 90 °C. DMF: *N*,*N*-dimethylformamide; NBS: *N*-bromosuccinimide; AIBN: azobisisobutyronitrile; dppf: 1,1′-bis(diphenylphosphanyl)ferrocene; DMSO: dimethylsulfoxide.

Fluorescence binding studies: Since the compounds were designed to change their fluorescence properties upon sugar binding, we studied their fluorescence properties under various conditions. However, before such studies, we wanted to make sure that these boronic acids have sufficient water solubility for the study. Therefore, we took seven UV spectra of each boronic acid in a concentration range of 1×10^{-4} (1% methanol in phosphate buffer, pH 7.4) to 1×10^{-6} M. Good linear relationships between concentrations and UV absorbance were obtained for all four boronic acids, a result indicating that these compounds were completely soluble and aggregation was not an issue under the conditions studied. As a result, we selected a concentration range of 1×10^{-5} to 5×10^{-6} M for the binding tests at pH 7.4 (1% MeOH in phosphate buffer).

The boronic acids themselves showed an excitation wavelength (λ_{ex}) of 493 nm and an emission wavelength (λ_{em}) of 567 nm. Upon binding with a model sugar, fructose, the emission blue shifted to 550 nm and the fluorescence intensity increased by about 2-fold for all these boronic acids (Figure 1). Subsequent extensive binding studies were then conducted at a sensor concentration of 1×10^{-5} M for boronic acids **1a**, **1b**, and **1d** and 5×10^{-6} M for boronic acid **1c**.

The emission shift and the fluorescence intensity changes of the boronic acids upon binding with sorbitol followed the same trend as those with fructose, and the fluorescence intensity increased to an even larger degree (about fourfold; Figure 2). Such results are consistent with our earlier observations that that carbohydrates that bind more tightly tend to bring on greater maximal fluorescence intensity changes.[47–52] Similar studies were conducted with glucose.

The apparent association constants (K_a) between the boronic acids and the three sugars were determined. The affinity trend with the boronic acids followed the order D-sorbi-

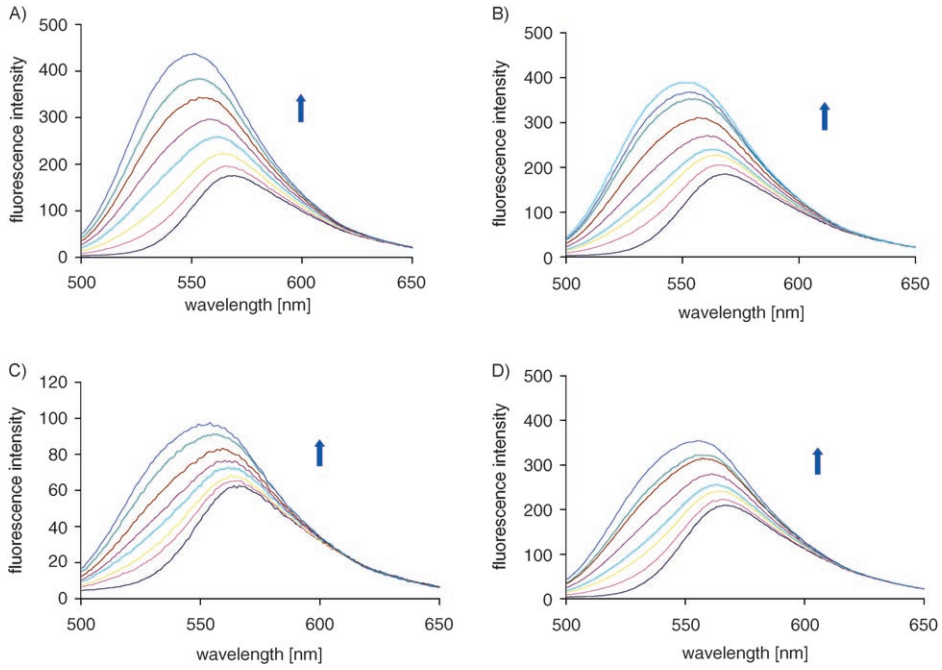


Figure 1. Fluorescence spectra of boronic acids upon addition of D-fructose (0–500 mM) in 0.1 M phosphate buffer at pH 7.4 with 1% MeOH; $\lambda_{ex}=493$ nm. A) **1a** (1×10^{-5} M); B) **1b** (1×10^{-5} M); C) **1c** (5×10^{-6} M); D) **1d** (1×10^{-5} M).

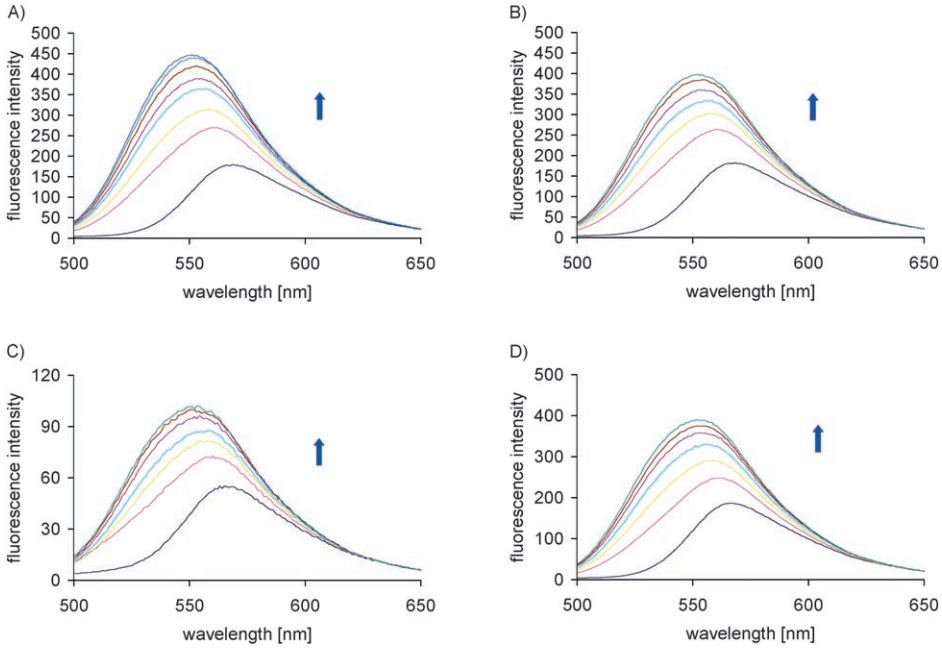


Figure 2. Fluorescence spectra of boronic acids upon addition of D-sorbitol (0–500 mM) in 0.1 M phosphate buffer at pH 7.4 with 1% MeOH; $\lambda_{ex}=493$ nm. A) **1a** (1×10^{-5} M); B) **1b** (1×10^{-5} M); C) **1c** (5×10^{-6} M); D) **1d** (1×10^{-5} M).

tol > D-fructose > D-glucose, which is consistent with previous literature reports for other monoboronic acids (Table 1).[1,4] Such intrinsic preference for different diols by monoboronic acids dictates the need for more than a single boronic acid in the construction of selective sensors for various carbohydrates. It is interesting to note that the four analogues with varying substituents on the phenylboronic acid moiety showed very similar binding constants (Table 1). Such results further confirm our earlier findings that the substituent effect on the binding affinity of a phenylboronic acid moiety is hard to predict,[4] especially when there is an amino group in a 1,5 relationship.[19]

The newly synthesized compounds (**1b–d**) showed very similar properties to those of the original compound **1a**. It should be noted that, in our previous work, methanol concentrations affected the binding constants of **1a**. For example, the binding constant of **1a** with fructose was $57\ M^{-1}$ in 0.1% methanol/buffer solution and changed to $25\ M^{-1}$ in 1% methanol/buffer.[13] However, the binding constants with other sugars showed little change with this minor change in methanol concentration.

pH titration and apparent pK_a determination: With the aim of understanding the basic mechanism through which fluorescence intensity changes occur, we studied the pH profiles of the fluorescence intensity in the absence and presence of sugars (500 mM). When tested in the absence of any sugar, the emission intensity of **1a** increased nine-fold at 550 nm upon changing the pH value from 2 to 12 (Figure 3), with an apparent pK_a value of about 6.0, which was assigned to the boronic acid moiety (Table 2).

When the pH value was changed from 2 to 12, the fluorescence intensity of **1a** at 550 nm increased 33-, 11-, and 40-fold with the addition of fructose, glucose, and sorbitol, respectively. The apparent pK_a values observed were 3.9, 5.5, and 4.1 for the esters of fructose, glucose, and sorbitol, respectively. The apparent pK_a values of **1a** in 1% methanol/buffer were slightly different from those of previous

Table 1. Apparent association constants (K_a) of the boronic acids with different sugars.[a]

	Fructose		Glucose		Sorbitol	
	K_a [M^{-1}]	ΔI_f[b] (fold)	K_a [M^{-1}]	ΔI_f[b] (fold)	K_a [M^{-1}]	ΔI_f[b] (fold)
1a[c]	25±2	2.5	1.4±0.3	1.1	110±1	2.6
1b[c]	28±1	2.4	1.1±0.2	1.1	117±3	2.5
1c[d]	27±1	1.7	1.4±0.2	1.2	103±2	2.2
1d[c]	24±2	1.8	0.9±0.3	1.1	98±6	2.4

[a] Experiments in duplicate were conducted in 0.1 M buffer solution at pH 7.4 with 1% MeOH. [b] ΔI_f[a] (fold): represents maximum intensity change over initial intensity. [c] [boronic acid] = 1×10^{-5} M. [d] [boronic acid] = 5×10^{-6} M.

Table 2. Apparent pK_a values of the boronic acids in the absence and presence of sugars.[a]

pK_a	Boronic acid	Fructose	Glucose	Sorbitol
1a[b]	6.0±0.2	3.9±0.4	5.5±0.3	4.1±0.3
1b[b]	6.3±0.2	4.2±0.2	4.9±0.2	3.9±0.1
1c[c]	5.6±0.1	3.7±0.2	4.7±0.1	3.6±0.2
1d[b]	5.8±0.1	3.6±0.1	5.5±0.3	3.5±0.3

[a] All experiments were performed in duplicate; sugar concentration: 500 mM in 0.1 M buffer solution at pH 7.4 with 1% MeOH. [b] [boronic acid] = 1×10^{-5} M. [c] [boronic acid] = 5×10^{-6} M.

studies in 0.1% methanol/buffer.[13] The trend of the apparent pK_a values with the other three boronic acids (**1b–d**) followed the order boronic acid alone > D-glucose > D-fructose > D-sorbitol (Table 2), as expected.[1,4,13] These apparent pK_a values showed a general Hammett correlation, as expected from our earlier publication (data not shown).[4]

The shift to the left in the pH-titration traces (Figure 3) for the sorbitol and fructose esters compared with those for the glucose ester and boronic acids alone is consistent with previous findings that sorbitol and fructose esters have lower apparent pK_a values than the free boronic acid and the glucose ester.[1,4,13] The overall lower apparent pK_a values for those compounds with an electron-withdrawing group (**1c** and **1d)** than for those with no such substituents or with an electron-donating substituent (**1a** and **b**) is to be expected.[4]

In order to achieve a good understanding of the origin of the fluorescence intensity changes, we took **1a** as an example to study the pH profile of UV absorbance in the absence and presence of fructose (500 mM). For boronic acid **1a** alone, the λ_{max} value changed from 440 to 492 nm when the solution pH value increased from 4.5 to 7.5; the UV intensity decreased by half at 440 nm and increased 3-fold at 493 nm. For the boronic ester of **1a** with fructose, the UV wavelength changed from 440 nm to 479 nm when the solution pH value increased from 2.5 to 5.0; the absorbance intensity showed a slight decrease in the wavelength range 400–425 nm and the intensity increased 2-fold at 479 nm (Figures 4 and 5). Such results suggest that the observed fluorescence intensity changes upon variation in the pH value could, at least partially, be attributed to the increased absorption at the excitation wavelength. The apparent pK_a values of **1a** and the **1a** ester form were 6.0 and 3.9 from the UV studies; these values are consistent with the results of the fluorescence studies (Figure 3 and Table 2). As shown in Figure 4 and 5B, the UV λ_{max} value of **1a** changed from 492 to 479 nm after fructose addition at a near physiological pH value.

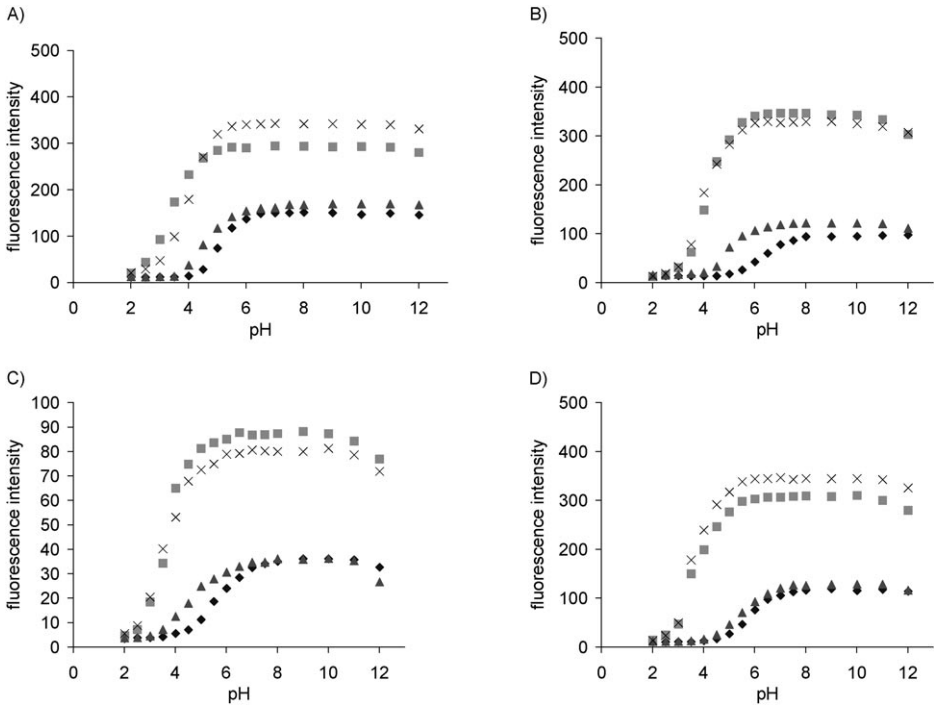


Figure 3. pH profile of the fluorescence intensity of boronic acids in the absence and presence of sugars in 0.1 M aqueous phosphate buffer with 1% MeOH, [sugar] = 500 mM, λ_{ex} = 493 nm, λ_{em} = 550 nm. Boronic acid alone (♦), in the presence of D-fructose (■), in the presence of D-glucose (▲), and in the presence of D-sorbitol (×). A) **1a** (1×10^{-5} M); B) **1b** (1×10^{-5} M); C) **1c** (5×10^{-6} M); D) **1d** (1×10^{-5} M).

Fluorescence quantum yield studies: The fluorescence quantum yields for boronic acids **1a–d** and their sugar complexes were also determined with fluorescein as a reference compound.[53,54] The quantum yields of the boronic acids were determined according to Equation (1), where Q is quantum yield, A is UV absorbance, OD is optical density (fluorescence), and subscript R indicates the reference compound.[55,56]

$$Q = Q_R(A/A_R)(OD_R/OD) \quad (1)$$

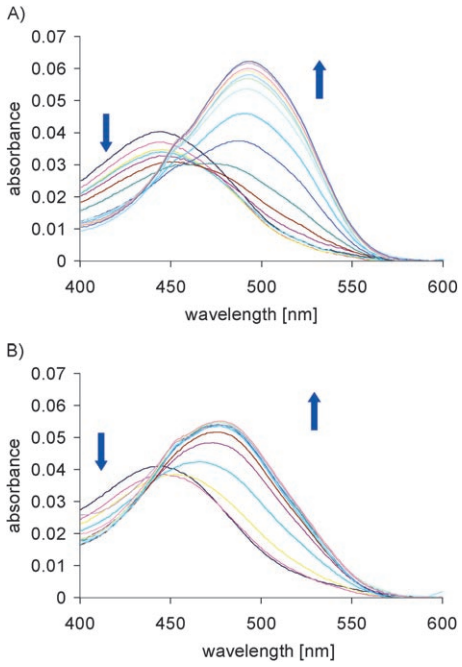


Figure 4. UV spectra of pH studies of boronic acid **1a** (1×10^{-5} M) in the absence and presence of fructose in 0.1 M aqueous phosphate buffer with 1% MeOH and pH value changes from 2.0 to 12.0. A) Boronic acid **1a** alone; B) **1a** in the presence of D-fructose (500 mM). All experiments were performed in duplicate.

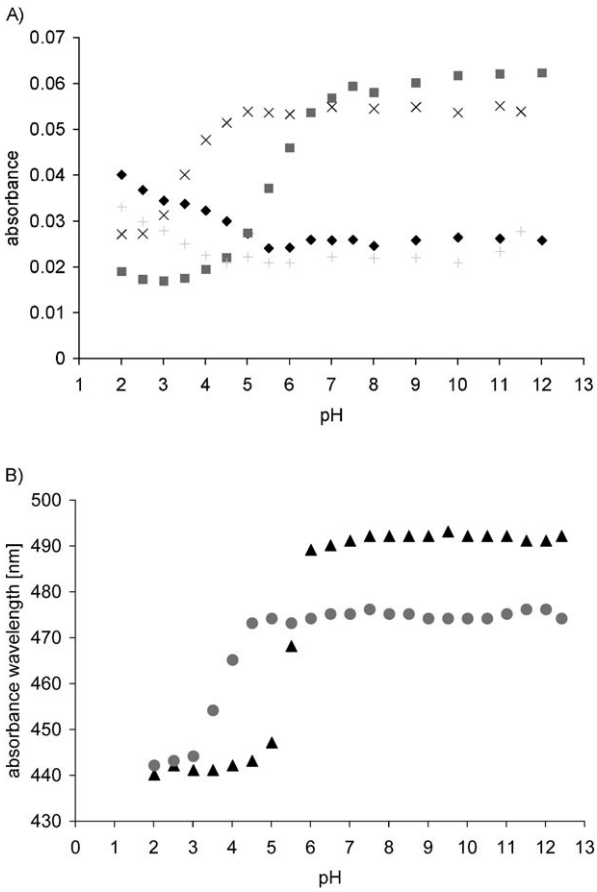


Figure 5. pH profile of the UV absorbance λ_{max} and intensity of boronic acid **1a** (1×10^{-5} M) in the absence and presence of fructose in 0.1 M aqueous phosphate buffer with 1% MeOH and [fructose] = 500 mM. A) Absorbance intensity changes with varying pH. Boronic acid alone at 440 nm (◆), boronic acid alone at 493 nm (■), in the presence of D-fructose (500 mM) at 415 nm (+), and in the presence of D-fructose (500 mM) at 479 nm (×); B) absorbance λ_{max} changes with different pH values. Boronic acid alone (▲) and in the presence of D-fructose (500 mM; ●). All experiments were performed in duplicate.

The results showed that the quantum yields of the boronic acids in the presence of fructose and sorbitol were higher than those of the boronic acids alone and in the presence of glucose, a fact that is consistent with the trend of fluorescence intensity changes (Table 3). However, these quantum yields are not directly correlated with the apparent pK_a value of each compound. This is understandable since many other factors, such as flexibility, solvation, and excited-state electron-density distribution, are expected to affect the quantum yields of these compounds as well.

Solvent studies and proposed mechanism for the fluorescence property changes: In order to understand the reason for the observed spectroscopic property changes upon sugar addition and variation of the pH value, we also studied solvent effects on boronic acid **1b** and a reference compound **3**. When the solvent polarity decreased from water to ethyl acetate, a significant blue shift in the λ_{max} value and the emission wavelength for both compounds was observed (Tables 4 and 5). Furthermore, both the UV and fluorescence intensities increased with decreases in the solvent polarity. Such results are consistent with an excited-state internal charge-transfer (ICT) mechanism,[15,57–65] in which polar solvents help to stabilize the excited-state charge separation and therefore lower the energy gap between the ground and excited states.[65,66] Other possibilities may also include a change in the specific species formed when going from an aqueous environment to an organic solution.[10,40,41,43,67–70]

Table 3. Fluorescence quantum yields of the boronic acids alone and in the presence of various sugars.[a]

	Boronic acid	Fructose	Glucose	Sorbitol
1a[b]	0.026 ± 0.003	0.081 ± 0.003	0.035 ± 0.004	0.084 ± 0.005
1b[b]	0.037 ± 0.002	0.095 ± 0.002	0.052 ± 0.001	0.101 ± 0.001
1c[c]	0.048 ± 0.002	0.090 ± 0.004	0.059 ± 0.001	0.103 ± 0.007
1d[b]	0.038 ± 0.003	0.093 ± 0.002	0.053 ± 0.002	0.093 ± 0.002

[a] Sugar concentration: 500 mM in 0.1 M buffer solution at pH 7.4 with 1% MeOH; all experiments were performed in duplicate. [b] [boronic acid] = 1×10^{-5} M. [c] [boronic acid] = 5×10^{-6} M.

Table 4. Solvent effect on the spectroscopic properties of compound **3**.[a]

	H_2O	MeOH	Ethyl acetate
λ_{max} [nm]	434 ± 1	433 ± 1	417 ± 2
UV intensity[b]	1.0	2.6 ± 0.3	2.9 ± 0.2
emission wavelength [nm]	542 ± 1	527 ± 1	494 ± 1
emission intensity relative to that in water	1.0	6.9 ± 0.2	10.6 ± 0.4

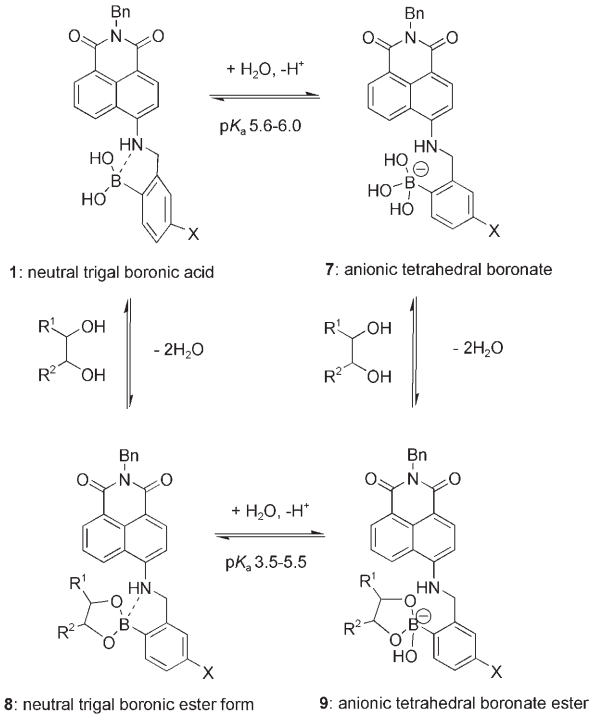
[a] [compound **3**] = 1×10^{-5} M. All experiments were performed in duplicate. [b] Relative to that in water.

Table 5. Solvent effect on the spectroscopic properties of **1b**.[a]

	H_2O	MeOH	Ethyl acetate
color	red	orange	yellow
λ_{max} [nm]	492±1	486±2	466±3
UV intensity[b]	1.0	1.3±0.1	1.4±0.1
emission wavelength [nm]	567±1	559±1	537±1
emission intensity relative to that in water	1.0	5.3±0.2	14.4±1.1

[a] [boronic acid **1b**] = 5×10^{-6} M. All experiments were performed in duplicate. [b] Relative to that in water.

The similar solvent effects on compound **3**, which does not have a boronic acid moiety, and boronic acid **1b** indicate that the phenylboronic acid moiety itself does not directly participate in the generation of fluorescence, although the formation of the anionic boronate species (Scheme 3) may influence the fluorescence properties of **1b** (and **1a**, **1c**, and **1d**), which will be discussed in the next section. The similar behaviors among **1a–d** in terms of λ_{max}, emission wavelength, pH profiles, and sugar-induced fluorescence changes also suggest that the phenylboronic acid moiety plays only an auxiliary role in fluorescence modulation and is not part of the fluorophore that is responsible for fluorescence generation.



Scheme 3. Equilibrium between different forms of boronic acids **1a–d** in the absence and presence of sugars at different pH values.

The proposed internal charge-transfer mechanism is also consistent with the pH profiles presented in Figure 4. As the pH value increases, the boronic acid moiety becomes ionized, which gives a net −1 charge. The positioning of a charged functional group next to the fluorophore should help to stabilize the excited-state charge separation and should result in a bathochromic shift of the UV spectra with increased intensity. To a certain extent, the effect of the negatively charged boron atom is similar to increased solvent polarity on the stabilization of the excited-state charge separation.

Through a combination of all of the results stated above, one can summarize the structural changes of **1a–d** and their relationship to the fluorescence properties by using Scheme 3. At low pH values, boronic acids **1a–d** exist in the neutral trigonal form, **1**, with a possible B–N bond through donation of the nitrogen lone-pair electrons to the boron open shell.[40,41,43,71] It is understood that the aromatic amine group involved is not a strong Lewis base because of the substitution of two electron-withdrawing imide functional groups on the naphthalene ring. Therefore, the exact strength (or existence) of the B–N bond is not clear. However, in either case, it does not affect the subsequent analysis of the observed fluorescence changes. As the solution pH value increases toward the respective pK_a values (5.6–6.0; Table 2), the boron atom is converted into its anionic tetrahedral form, **7**. As discussed earlier, this generation of a net negative charge helps to stabilize the excited-state charge transfer and therefore causes a bathochromic shift of the UV λ_{max} value with increased intensity (Figures 4 and 5). The increased absorbance can be at least a partial contributing factor for the observed fluorescence intensity increase. With the addition of a carbohydrate, the pK_a value of the boronic ester **8** becomes even lower than that of the boronic acid species (Table 2).[1,2,4] Similarly, an increased pH value would result in the generation of the anionic boronate ester **9**, which should also help to stabilize the excited-state charge transfer in much the same way as the boronate species **7** and would explain the bathochromic shift in the UV λ_{max} value. As presented in Figures 1–3 and Tables 1 and 3, the boronate esters **9** are much more fluorescent than the corresponding boronates **7**. Complexation with a sugar will most likely add steric hindrance and decrease the freedom of rotation around the C–N bond of the aniline nitrogen atom, which can increase the fluorescence quantum yield and consequently the fluorescence intensity. Sugar addition to the boronic acid solution at the physiological pH value also causes a hypsochromic shift in both the UV λ_{max} value (Figures 4 and 5B) and fluorescence emission wavelength (Figures 1 and 2). Since at the physiological pH value, both the boronic acid **1** and the boronic ester **8** should be in their respective ionized states, **7** and **9**, there is no difference in their net charge. Therefore, the observed hypsochromic shift is most likely due to the perturbation of the position and/or orientation of the anionic boronate esters **9** relative to that of the boronate species **7**. It is reasonable to expect that addition of a sugar moiety would increase steric hindrance, which in turn could increase the distance between the anionic boronate functional group and the fluorophore. The distance between the boron and aniline nitrogen atoms is especially important because the lone-pair electrons of the nitro-

gen atom are most likely involved in the excited-state charge transfer.[72–77] If this is indeed the case, the increased B–N distance (this is simply the B–N distance, not a B–N bond length as there is no B–N bond here) could lessen the stabilizing effect of the negatively charge boronate in **9** on the excited-state charge-transfer species, which in turn should lead to a hypsochromic shift.

To examine structural aspects of the sugar–boronic acid complex, we performed a series of density function theory (DFT)[78,79] calculations in a polarizable continuum model (PCM)[80] to investigate the structures of these boronic acids and the corresponding esters with fructose. Electronic structure calculations were performed by using the Gaussian 03 series of programs[81] on a Linux-based 40-node cluster. The DFT method B3LYP and the 6–31+G(d,p) base set was used for all calculations, along with the PCM solvation model. The PCM solvation model was used in single-point energy calculations (PCM(sp)) and during geometry optimizations and frequency calculations (PCM(opt)). All calculations with the PCM solvent model employed the united-atom topological model for Hartree–Fock (UAHF) atomic radii during construction of the solvent cavity, as recommended in the Gaussian 03 user's reference manual. All geometries were fully optimized, and the characters of the stationary points found were confirmed by a harmonic frequency calculation at the same level of theory to ensure a minimum was located. The results are shown in Table 6. Figure 6 shows the minimized structures of **1a** in different forms and the B–N distances.

Table 6. Calculated B–N distances of boronic acids **1a–d** in the neutral form (**1**), anionic form (**7**), and complexed boronate ester form (**9**).

	B–N distance [Å]		
	neutral	anionic	complex
1a	1.85	3.19	3.33
1b	1.85	3.02	3.31
1c	1.91	3.05	3.31
1d	1.84	3.01	3.17

The B–N distance for a strong B–N bond is usually around 2 Å.[82–84] In the neutral trigonal form (**1**, Scheme 3), the calculated B–N bond lengths of **1a–d** were 1.84–1.91 Å; these values suggest the possibility of B–N interactions to some degree. Furthermore, the solvent-bridging effect observed in the crystal state for some boronic acids is another factor that could influence the interpretation of the B–N bond strength and/or existence.[43] It should be noted that our group recently reported the reexamination of the B–N bond issue and concluded that B–N bond hydrolysis after complexation with a diol was the dominate pathway in most cases in an aqueous environment.[40,41] Such results were further supported by studies from the Anslyn group[43] and are consistent with the present study. Upon conversion of the boron atom into its anionic form **7**, the B–N distances increased to about 3 Å. This is understandable since the tetrahedral form of the boronic acid functional group has no

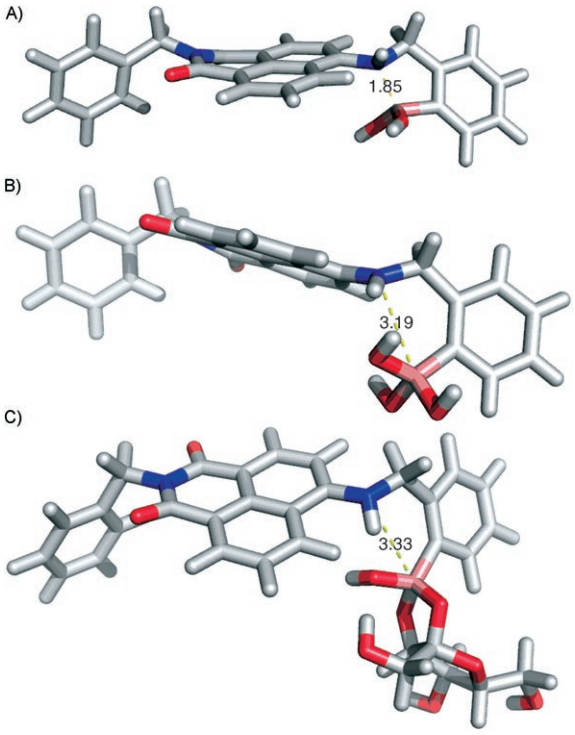


Figure 6. Modeled structures of boronic acid **1a** in the neutral form (A), the anionic form (B), and the boronic ester form (C).

open shell to accommodate the lone-pair electrons of the nitrogen atom and therefore there cannot be B–N bond formation. The calculated B–N distances in boronate fructose complexes **9** with **1a–d** were 0.13–0.29 Å longer than those in the corresponding boronates **7**. The lengthened B–N distance should result in a lessened stabilization of the excited-state charge separation and then a hypsochromic shift. Therefore, the computational results are consistent with the observed hypsochromic shifts of the UV λ_{max} value and fluorescence emission wavelength as discussed above.

Conclusion

We have synthesized and evaluated a series of long-wavelength (around 570 nm) boronic acid fluorescent reporters, which showed good water solubility and significant fluorescence changes upon sugar addition. Variation of the *para*-position substituents on the phenylboronic acid moiety had very little effect on either the binding affinity or the spectroscopic properties. Mechanistic studies suggest that these fluorophores involve excited-state charge transfer, which can be stabilized by the appended boronic acid group once it is ionized. Such stabilization is consistent with the observed fluorescence and UV pH profiles. The increased fluorescence intensities of these boronic acids upon sugar addition can be explained by the increased B–N distance in the boronate complex state, **9**; this conclusion is supported by extensive computational results. The boronic acids synthesized

(**1a–d**) will be very useful for the preparation of long-wavelength sensors and boronolectins[29] for carbohydrates, glycolipids, and glycoproteins. Work on incorporating these boronic acids into DNA for fluorescent aptamer development is in progress.[85]

Experimental Section

General: ^{1}H and ^{13}C NMR spectra were recorded on a Bruker 400 MHz spectrophotometer in deuterated choloroform ($CDCl_3$) or deuterated [D_6]DMSO ($(CD_3)_2SO$) with either tetramethylsilane (TMS; $\delta=0.00$ ppm) or the NMR solvent as the internal reference unless otherwise specified. HPLC purification was carried out with a Shimadzu LC-10AT VP system and a Zobax C18 reversed-phase column (4.6 mm×25 cm). Fluorescence spectra were recorded on a Shimadzu RF-5301 PC spectrofluorometer. Absorption spectra were recorded on a Shimadzu UV-1700 UV/Vis spectrophotometer. Quartz cuvettes were used in all fluorescence and UV studies. All pH values were determined by a UB-10 Ultra basic benchtop pH meter (Denver Instruments). Analytical thin-layer chromatography (TLC) was performed on Merck silica gel 60 plates (0.25 mm thickness with F-254 indicator). Sugars, buffers, and diols were bought from Aldrich or Acros and were used as received. Water used for the binding studies was doubly distilled and further purified with a Milli-Q filtration system. Solvents for extraction and chromatography were used as received. Drying solvents (DMF, DMSO) were purchased from Acros. ^{1}H, ^{13}C NMR and MS spectra of selected compounds are given in the Supporting Information.

General procedure for synthesis of compounds 5: Dry CCl_4 (100 mL) was added to a mixture of **4** (10 mmol, 1.0 equiv), AIBN (0.5 mmol, 0.05 equiv), and NBS (10 mmol, 1.0 equiv). The mixture was heated at 70 °C (reflux) under white light (IR: 115–125 V, 175 W) with stirring for 2.5 h. Then solvent was evaporated under vacuum.

4-Bromo-3-bromomethylbenzoic acid methyl ester (5c): Column chromatography (hexanes/ethyl acetate 8:1) gave **5c** (38%) as white crystals. TLC (hexanes/ethyl acetate 10:1): $R_f=0.50$; ^{1}H NMR ($CDCl_3$): $\delta=8.08$ (s, 1H), 7.77 (t, $J=8.4$ Hz, 1H), 7.62 (d, $J=8.4$ Hz, 1H), 4.58 (s, 2H; CH_2), 3.89 ppm (s, 3H; CH_3); ^{13}C NMR ($CDCl_3$): $\delta=165.8$, 137.5, 133.6, 132.2, 130.8, 130.1, 129.8 ($COOCH_3$), 52.5 (OCH_3), 32.6 ppm (CH_2); MS (ESI): m/z (%): 310 [M^+].

1-Bromo-2-bromomethyl-4-fluorobenzene (5d): Column chromatography (hexanes) gave **5d** (39%) as white crystals. TLC (hexanes/ethyl acetate 10:1): $R_f=0.50$; ^{1}H NMR ($CDCl_3$): $\delta=7.48$–7.52 (m, 1H), 7.16–7.19 (m, 1H), 6.86–6.91 (m, 1H), 4.52 ppm (s, 2H; CH_2); ^{13}C NMR ($CDCl_3$): $\delta=163.1$, 160.6, 138.9, 134.5, 118.1, 117.3, 32.5 ppm (CH_2); MS (ESI): m/z (%): 268 [M^+], 270 [$M+2H$]$^+$.

1-Bromo-2-bromomethyl-4-nitrobenzene (5e): Column chromatography (hexanes/ethyl acetate 20:1) gave **5e** (21%) as white crystals. TLC (hexanes/ethyl acetate 20:1): $R_f=0.50$; ^{1}H NMR ($CDCl_3$): $\delta=8.31$ (s, 1H), 8.00 (m, $J=11.6$ Hz, 1H), 7.76 (d, $J=8.4$ Hz, 1H), 4.61 ppm (s, 2H; CH_2); ^{13}C NMR ($CDCl_3$): $\delta=139.0$, 134.5 (2C), 131.6, 125.9, 124.4, 31.5 ppm (CH_2); MS (ESI): m/z (%): 295 [M^+], 297 [$M+2H$]$^+$.

General procedure for synthesis of compounds 6: Dry DMF (25 mL) was added to a mixture of **3** (1.00 mmol, 1.0 equiv) and sodium hydride (60%, 1.03 mmol, 1.03 equiv). The mixture was stirred for 10 min, then a solution of **5** (1.00 mmol, 1.0 equiv) in anhydrous DMF (15 mL) was added dropwise under N_2 at room temperature with stirring. Afterwards, the stirring was continued at room temperature for 12 h. The solvent was then evaporated under vacuum.

2-Benzyl-6-(2-bromo-5-methoxybenzylamino)-benzo[*de*]isoquinoline-1,3-dione (6b): Column chromatography (hexanes/ethyl acetate 12:1) gave **6b** (31%) as a yellow powder. TLC (hexanes/ethyl acetate 1:2): $R_f=0.50$; ^{1}H NMR ($CDCl_3$): $\delta=8.64$ (d, $J=6.4$ Hz, 1H), 8.49 (d, $J=8.4$ Hz, 1H), 8.10 (d, $J=8.4$ Hz, 1H), 7.61–7.64 (m, 1H), 7.49–7.52 (m, 3H), 7.26–7.28 (m, 2H), 7.21 (d, $J=7.2$ Hz, 1H), 6.91 (d, $J=4.2$ Hz, 1H), 6.68–6.75 (m, 2H), 5.35 (s, 2H; CH_2), 4.63 (s, 2H; CH_2), 3.70 ppm (s, 3H; CH_3); ^{13}C NMR ($CDCl_3$): $\delta=176.6$, 175.3, 148.7, 141.3, 139.3, 137.8, 134.5, 133.9, 131.4, 128.8, 128.4, 127.2, 125.9, 125.1, 123.3, 120.8, 115.6, 114.5, 111.3, 105.2, 55.5 (OCH_3), 43.4 (CH_2), 29.7 ppm (CH_2); MS (ESI): m/z (%): 500.9 [$M-H$]$^+$, 501.8 [M^+], 502.8 [$M+H$]$^+$, 503.8 [$M+2H$]$^+$.

3-[(2-Benzyl-1,3-dioxo-2,3-dihydro-1H-benzo[*de*]isoquinolin-6-ylamino)-methyl]-4-bromobenzoic acid methyl ester (6c): Column chromatography (hexanes/ethyl acetate 8:1) gave **6c** (25%) as a yellow powder. TLC (hexanes/ethyl acetate 1:2): $R_f=0.45$; ^{1}H NMR ($CDCl_3$): $\delta=8.65$ (d, $J=$ .0 Hz, 1H), 8.48 (d, $J=8.4$ Hz, 1H), 8.18 (d, $J=7.6$ Hz, 1H), 8.07 (s, 1H), 7.89 (m, $J=8.4$ Hz, 1H), 7.76 (d, $J=8.4$ Hz, 1H), 7.69 (t, $J=7.6$ Hz, 1H), 7.55 (d, $J=6.8$ Hz, 2H), 7.23 (m, 3H), 6.71 (d, $J=8.4$ Hz, 1H), 5.39 (s, 2H; CH_2), 4.77 (s, 2H; CH_2), 3.88 ppm (s, 3H; CH_3); MS (ESI): m/z (%): 529.0 [M^+], 531.0 [$M+2H$]$^+$.

2-Benzyl-6-(2-bromo-5-fluorobenzylamino)-benzo[*de*]isoquinoline-1,3-dione (6d): Column chromatography (hexanes/ethyl acetate 12:1) gave **6d** (27%) as a yellow powder. TLC (hexanes/ethyl acetate 1:2): $R_f=0.50$; ^{1}H NMR ([D_6]acetone, 400 MHz): $\delta=8.72$ (d, $J=8.4$ Hz, 1H), 8.57 (d, $J=7.2$ Hz, 1H), 8.37 (d, $J=8.4$ Hz, 1H), 7.79–7.72 (m, 3H), 7.46 (d, $J=$ 7.2 Hz, 2H), 7.31–7.20 (m, 4H), 7.11–7.06 (m, 1H), 6.71 (d, $J=8.4$ Hz, 1H), 5.33 (s, 2H; CH_2), 4.81 ppm (s, 2H; CH_2); ^{13}C NMR ([D_6]acetone): $\delta=178.2$, 176.4, 163.3, 149.7, 138.4, 134.5, 134.1, 130.9, 128.2, 127.6, 126.9, 124.9, 120.8, 116.0, 115.7, 110.4, 104.8, 47.2 (CH_2), 42.7 ppm (CH_2); MS (ESI): m/z (%): 489.0 [M^+], 490.1 [$M+H$]$^+$.

General procedure for synthesis of compounds 1: According to a typical borylation procedure, **6** (0.237 mmol, 1.0 equiv), bis(neopentylglycol) borane (64.2 mg, 0.284 mmol, 1.2 equiv), [$PdCl_2$(dppf)] (23.5 mg, 0.029 mmol, 0.12 equiv), and potassium acetate (70.5 mg, 0.718 mmol, 3 equiv) were mixed at room temperature under an N_2 atmosphere. This was followed by addition of anhydrous DMSO (2 mL) with a syringe. The solution was heated at 90 °C for 10 h and then cooled to room temperature. Ethyl acetate (20 mL) and water (15 mL) were added to the reaction mixture. The separated organic phase was washed with water (2× 10 mL). After drying over sodium sulfate and solvent evaporation, the residue was purified by column chromatography (hexanes/ethyl acetate 3:2, to ethyl acetate/methanol 10:1) and further purified by HPLC (C18 RP column). Elution conditions: CH_3CN/MeOH (1 mL min^{-1}); 0–10 min (CH_3CN 100%), 10–20 min (CH_3CN 100→0%), 20–29 min (CH_3CN 0%), 29–30 min (CH_3CN 0→100%).

2-Benzyl-6-(2-boronic acid-5-methoxy-benzylamino)-benzo[*de*]isoquinoline-1,3-dione (1b): **1b** (24%) was obtained as a red powder. HPLC: $t_R=$ 20 min; ^{1}H NMR (CD_3OD, 400 MHz): $\delta=8.22$ (d, $J=7.2$ Hz, 1H), 8.05 (d, $J=8.4$ Hz, 1H), 7.59 (d, $J=6.8$ Hz, 1H), 7.35 (d, $J=8.4$ Hz, 2H), 7.17–7.27 (m, 4H), 7.02 (d, $J=7.6$ Hz, 2H), 6.74 (t, $J=10.8$ Hz, 1H), 6.17 (d, $J=8.4$ Hz, 1H), 5.33 (s, 2H; CH_2), 4.52 (s, 2H; CH_2), 3.73 ppm (s, 3H; CH_3); ^{13}C NMR (CD_3OD): $\delta=166.3$, 164.6, 163.2, 159.8, 141.8, 138.6, 137.3, 132.0, 129.3, 128.7, 127.8, 127.6, 127.2, 126.3, 124.8, 119.6, 117.9, 112.5, 111.8, 102.3, 100.3, 54.2 (OCH_3), 46.3 (CH_2), 42.5 ppm (CH_2); MS (ESI): m/z (%): 466.2 [M^+], 467.2 [$M+H$]$^+$, 468.2 [$M+2H$]$^+$.

3-[(2-Benzyl-1,3-dioxo-2,3-dihydro-1*H*-benzo[*de*]isoquinolin-6-ylamino)-methyl]-4-boronic acid-benzoic acid methyl ester (1c): **1c** (14%) was obtained as a red powder. HPLC: $t_R=22$ min; ^{1}H NMR (CD_3OD): $\delta=8.25$ (d, $J=6.8$ Hz, 1H), 8.15 (s, 1H), 8.08 (d, $J=8.4$ Hz, 1H), 7.88 (d, $J=$ 7.2 Hz, 1H), 7.72 (d, $J=8.0$ Hz, 1H), 7.62 (d, $J=6.8$ Hz, 1H), 7.36–7.43 (m, 3H), 7.26–7.29 (m, 2H), 7.20 (d, $J=8.8$ Hz, 1H), 6.18 (d, $J=8.8$ Hz, 1H), 5.35 (s, 2H; CH_2), 4.62 (s, 2H; CH_2), 3.88 ppm (s, 3H; CH_3); MS (ESI): m/z (%): 493.1 [$M-H$]$^+$, 495.1 [$M+H$]$^+$, 522.1 [$M-2H+2CH_3$]$^+$.

2-Benzyl-6-(2-boronic acid-5-fluorobenzylamino)-benzo[*de*]isoquinoline-1,3-dione (1d): **1d** (21%) was obtained as a red powder. HPLC: $t_R=$ 21 min; ^{1}H NMR (CD_3OD, 400 MHz): $\delta=8.55$ (d, $J=7.2$ Hz, 1H), 8.47 (d, $J=8.4$ Hz, 1H), 7.66 (d, $J=7.6$ Hz, 1H), 7.42–7.48 (m, 3H), 7.21–7.37 (m, 6H), 7.07–7.14 (m, 2H), 5.35 (s, 2H; CH_2), 4.55 ppm (s, 2H; CH_2); ^{13}C NMR (CD_3OD): $\delta=174.0$, 164.7, 164.2, 140.5, 137.67, 133.8, 130.12, 128.4, 128.0, 127.7, 126.8, 124.0, 123.0, 122.3, 114.4, 110.5, 107.4, 104.9, 48.3 (CH_2), 42.8 ppm (CH_2); MS (ESI): m/z (%): 453.1 [$M-H$]$^+$, 454.1 [M^+].

Procedures for the binding studies (with 1a as an example): Solutions of **1a** (1×10^{-5} M) and **1a** (1×10^{-5} M) with sugar (0.5 M) were prepared in 0.1 M phosphate buffer at pH 7.40. These two solutions were then mixed

in a 1-cm cuvette. In the solution, the ratio of boronic acid and sugar was increased gradually. After being shaken for 2 min, the solution was used to test the fluorescence intensity or UV absorbance immediately. Six to eight points were collected for the calculation of apparent binding constant, K_a, with the assumption of a 1:1 complex formation mechanism.

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