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Diol Appended Quenchers for Fluorescein Boronic Acid

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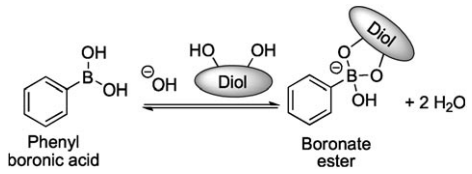
Dedicated to the 150th anniversary of Japan–UK diplomatic relations

Abstract: Fluorescein isothiocyanate is treated with 3-aminophenylboronic acid to provide a fluorescently tagged boronic acid derivative which is used to assess Förster resonance energy transfer (FRET) quenching upon boronate ester formation with a series of bespoke diol appended quenchers. Fluorescence spectroscopy comparison of quenching efficiency between treatment of fluorescein and its boronic acid appended congener with quencher appended diol reveals boronate ester formation (covalently linked) to be the more efficient regime and from the panel of quenchers which also included nucleosides.

Keywords: boronic acid · fluorophore · nucleoside · quencher · receptor

Introduction

Boronic acids readily and reversibly form boronate esters with 1,2- and 1,3-diols under basic conditions, Scheme 1 exemplifies cyclic boronate ester formation from phenyl boronic acid and a generic diol in the presence of hydroxide ions. Reversible cyclic boronate formation has been exploited widely in saccharide sensing[1–7] and supramolecular chemistry[8–10] as well as electrophoresis[11–13] and enantiomeric excess determinations,[14–17] the higher binding affinities of bidentate diols over monodentate alcohols permits studies to be performed in alcoholic solutions.[18]



Scheme 1. Generic phenylboronate ester formation.

It was reasoned that through judicious choice of boronic acid and diol derivatives in "molecular beacon" type fluorophore–quencher pairs, similar to the methodology used in quantitative PCR assays such as Taqman, may be covalently assembled in solution. That is since FRET efficiency is strongly dependent on donor–acceptor separation, covalently linking fluorophore and quencher by means of boronate ester formation should significantly enhance the observed quenching. This principle was recently demonstrated by us for surface appended fluorophores excited by means of the surface plasmon in an SPR experiment, for a highly engineered system with a specific target function.[19] Solution studies of this effect, represented generically in Scheme 2,

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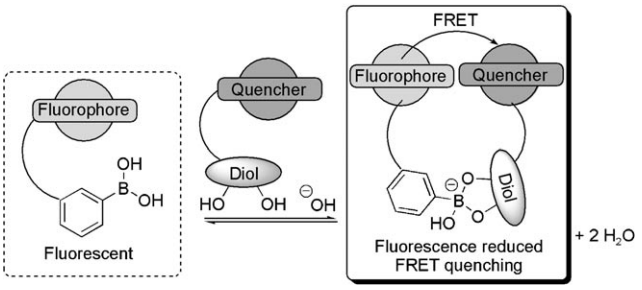


Scheme 2. Schematic representation of boronate ester formation resulting in an enhanced (static) FRET quenching.

with easy to prepare and operationally simple substrates are reported herein.

Results and Discussion

Synthesis

Derivatives of fluorescein **1** (depicted in Figure 1) are commonly used as biotags and are among the most readily available and easily accessible commercially available fluorophores. As such they are ideally suited to derivatization and

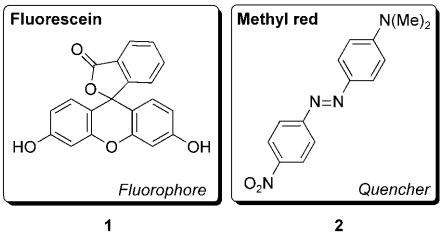
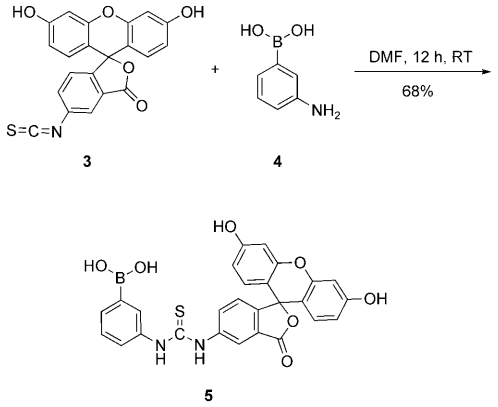


Figure 1. Structures of fluorescein **1** and methyl red **2**.

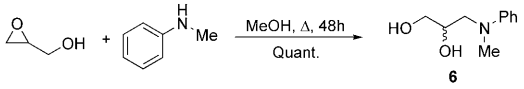
exploitation in various sensing and signalling regimes. Equally, that methyl red **2** is a routinely used quencher for fluorescein,[20] and the synthesis of derivatives is relatively trivial, renders it suitable for investigation in bespoke boronate diol signalling systems, as in Scheme 2.

First, a new boronic acid derivative of fluorescein was prepared as shown in Scheme 3. The NCS derivative of fluorescein **3** was stirred in dimethylformamide (DMF) with 3-aminophenylboronic acid **4**, at room temperature for 12 h to furnish the thiourea linked boronic acid fluorescein conjugate **5** in 68% yield.

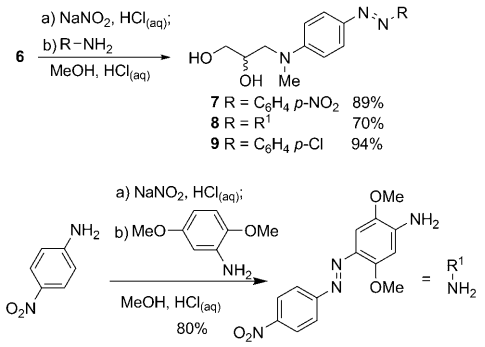


Scheme 3. Preparation of fluorescein boronic acid **5**.

Next, a series of diol appended quenchers, inspired by the methyl red motif, were prepared. Common to the synthesis of the quenchers prepared was diol **6**, which was prepared in quantitative yield, Scheme 4 To demonstrate the tunability of quencher design and ease of diol appended quencher synthesis, various aniline derivatives were employed in a diazotization step utilizing standard conditions to furnish diol appended quenchers **7**, **8**, and **9** in good yields, Scheme 5.



Scheme 4. Preparation of diol unit **6**.



Scheme 5. Synthesis of diol appended quenchers **7**, **8**, and **9** and preparation of intermediate to **8** (R^1–NH_2).

Abstract in Japanese:

今回我々は、フルオレセインイソチオシアネートと3-アミノフェニルボロン酸より蛍光タグ化されたボロン酸を合成した。合成したボロン酸は、消光剤が付加したジオールと反応させボロン酸エステルを形成させることにより、フェルスター蛍光共鳴エネルギー(FRET)を測定することに用いることが可能である。実際に、フルオルセインのみのものと、今回合成したフルオレセイン含有ボロン酸類に消光剤含有ジオールを反応させたものの蛍光強度を比較したところ、その蛍光強度の差は共有結合によるボロン酸エステルの形成により最も大きく影響を受け、それはヌクレオシドを含む種々の消光剤からの影響であることを明らかにした。

The absorbance spectra of the new diol appended quenchers **7**, **8**, and **9** where recorded and contrasted with that of methyl red **2** as well as the emission spectra of fluorescein **1** and the boronic acid derivative **5**, see Figure 2.

From Figure 2 it can be seen that the emission wavelength of fluorescein **1** matches the emission wavelength of its boronic acid appended congener **5**, thus confirming **1** is a suitable model for comparing interactions of **5** with respect to boronate ester formation. From comparison of the absorption maxima wavelengths of quenchers **2**, **7**, **8**, and **9** with fluorophores **5** and **1** in Figure 2, it can be seen that the best match between fluorescein's emission and quencher absorption is achieved with diol appended quencher **8**, decreasing in the order **7**, **9**, then **2**. Although quencher **8** offers the best match to fluorescein's emission, its absorbance spectrum displays the lowest maxima of the quenchers studied suggesting its efficiency as a quencher could be diminished with respect to **7** and **9**.

Fluorescence Quenching Experiments

Diol Appended Methyl Red Analogues

With a fluorophore appended boronic acid and a series of diol appended quenchers in hand, we set about assessing the influence of boronate ester formation between said quencher and fluorophore on quenching ability. That the role of the diol motif (and thus boronate ester formation) may be delineated, methyl red itself (**2**) was used as a control quencher. This means quenching observed as a result of the combination of **2** with **5** may be considered as the non-covalent (dynamic) quenching ability of methyl red. Thus, quenching over and above the non-covalent control should arise from an increased proximity of the quencher and enhanced FRET efficiency, in our case, as a result of boronate ester formation, that is, a covalent interaction results in an increased closeness in space (static), as depicted in Figure 3.

The presence of a quencher of suitable absorption characteristics should lead to a reduction in observed fluorescence intensity (I), thus from a Stern–Volmer plot of I_0/I as a function of quencher concentration one may compare relative quenching power for a given system (the greater the value

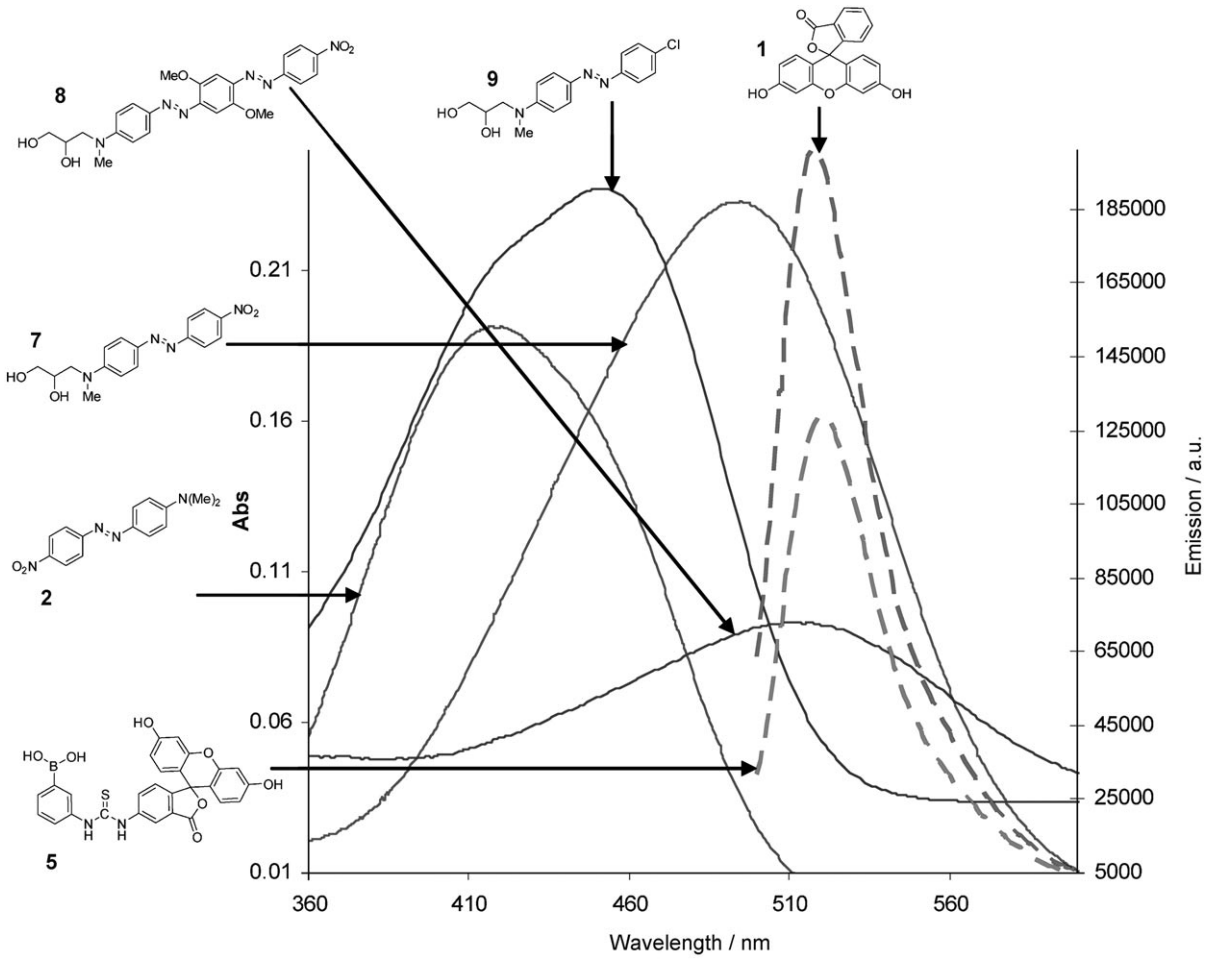


Figure 2. Absorbance (continuous lines) spectra of **2**, **7**, **8**, and **9** and emission (dotted lines) spectra of **1** and **5** ($\lambda_{ex}=490$ nm).

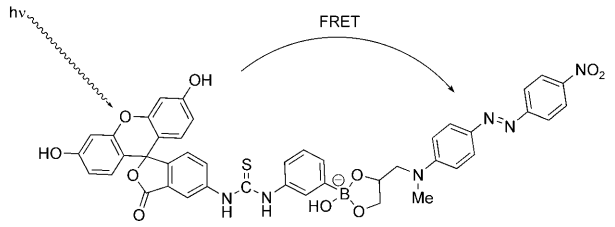


Figure 3. Proposed structure and FRET quenching mechanism of a boronate ester formed upon reaction of **5** with **7**.

of I_0/I the better the quencher). The fluorescence spectra of **5** ($\lambda_{ex}=490$ nm, 0.0135 μM, aqueous methanolic PBS, pH 8.2) was recorded and the obtained arbitrary intensity value of fluorescence at 520 nm, corresponding to the fluorescein fluorophore part of **5**, is the initial intensity of fluorescence (I_0). The effect of the presence of diol appended quenchers **7**, **8**, and **9** as well as methyl red **2** with no diol motif was assessed (0 to 15 μM). Figure 4 shows the relative quenching ability, I_0/I, upon the fluorescence of **5**.

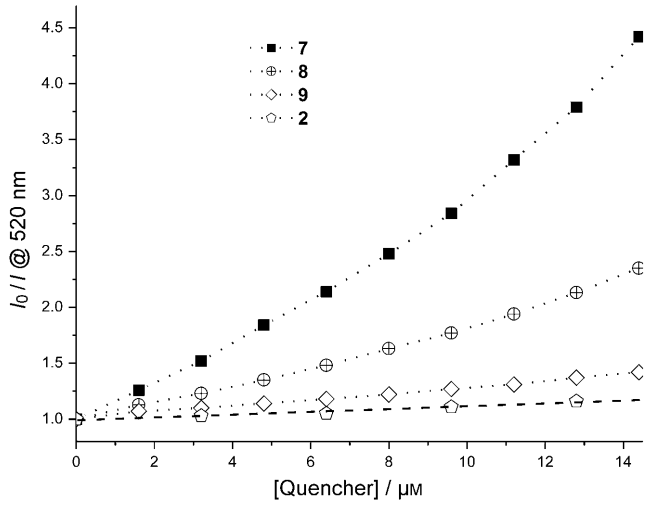


Figure 4. Comparison of relative quenching action observed on **5** (0.0135 μM) (I_0/I) upon addition of non-diol appended **2** and diol bearing units **7**, **8**, and **9** at 520 nm ($\lambda_{ex}=490$ nm).

From Figure 4, a plot of I_0/I reveals that, as expected, methyl red **2** (over the 0 to 15 μM range studied) had the least quenching effect on **5** (0.0135 μM). Gratifyingly, diol appended quenchers elicited a stronger quenching response. Whilst compound **9** gave only a slightly enhanced quenching effect over **2**, compounds **7** and **8** provided stronger quenching, with **7** being the better quencher of those tested under these conditions.

In Figure 4, diol and non-diol bearing quenchers are compared. Next, the effect of the boronic acid was probed by comparing the fluorescence quenching action of quenchers on fluorophores **1** and **5**. In two separate experiments, fluorescein **1** or boronic acid derivative **5** (0.0135 μM) were exposed to an increasing concentration of **7**, **8**, and **9** (0 to 15 μM). Quenching should be more extensive as a result of boronate ester formation owing to the increase proximity of the fluorophore quencher pair than in the system where no boronate ester formation is possible. Ratiometric comparison of the corresponding I_0/I plots is presented in Figure 5,

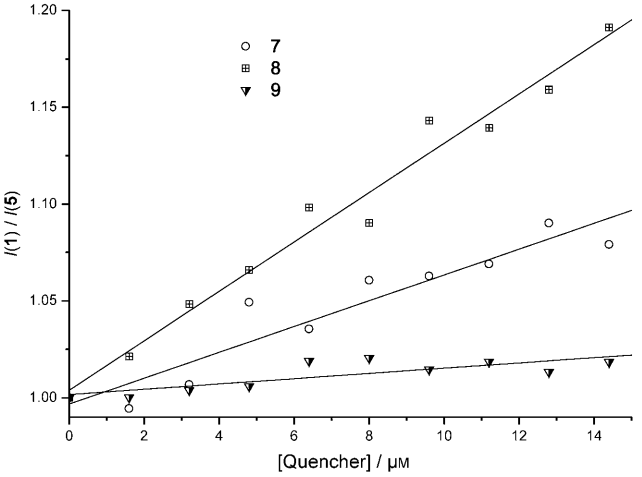


Figure 5. The enhancement in quenching as a result of the presence of a boronic acid in the fluorophore (I_0/I for **5**)/(I_0/I for **1**) (represented as I(**1**)/I(**5**)), at 0.0135 μM, for the addition of diol appended quenchers **7**, **8**, and **9**.

namely (I_0/I for **5**)/(I_0/I for **1**). Since both **5** and **1** would give rise to equivalent levels of dynamic quenching, it is reasonable to assume this comparison reveals the role of the boronic acid unit of **5**, which is responsible for initiating the static component of quenching, whereas **1** has little or no static component. Although a modified Stern–Volmer equation may be used to assess static and dynamic quenching,[21] we suggest our comparison of the corresponding I_0/I ratios is indicative of the inherent stability constant of the complexes formed. For clarity in graphical representations here and later in this paper we denote this value as I(**1**)/I(**5**) since I_0 was essentially the same in both cases, although (I_0/I for **5**)/(I_0/I for **1**) was calculated with the individual values for I_0 in each series.

The ratiometric comparison graphically represented in Figure 5 indicates that **8** has a better quenching ability than **7** when the effect of the presence of a boronic acid is probed (fluorescein **1** versus boronic acid derivative **5**), and **9** remains the poorest of the three diol appended quenchers compared. This apparent disparity with the data presented in Figures 4 and 5 may be explained by considering that although **7** is the better quencher of boronic acid appended fluorophore **5**, compound **8** has the greater difference between quenching ability in its action on **1** versus boronic acid **5**, which is consistent with the observation that **8** has a better match of absorption profile to fluorescein's emission but its absorption is not as efficient as that of **7**. Equally, this can explain why **9** is the poorest quencher of the three (least efficient overlap). As such, it may be inferred that

compound **8** has the highest binding affinity for **5** of the diol appended quenchers compared,[22] and is therefore the better quencher in terms of the relative difference with and without boronic acid.

Since compound **8** had the greatest ratiometric quenching enhancement between its use with boronic acid and non-boronic acid appended quenchers **1** and **5**, the effect of fluorophore concentration was also probed. From this series of experiments, an estimate of the role of boron and its ester formation with diol appended quenchers may be obtained. Using the Stern–Volmer data, the slopes of the lines obtained when I_0/I is plot against the volume of quencher **8** added, were determined for a range of fixed fluorophore concentrations (0.0135, 0.0270, 0.0405, 0.0540, 0.0675, 0.0810, 0.0945, 0.1080, 0.1215, and 0.1350 μM). In this case, I_0 was set at the first mixed systems fluorescence. This means I_0 corresponds to the fluorescence of systems containing 5 μM quencher and the difference in relative amounts of quencher represented as Δ [quencher], since it was found that inclusion of the zero quencher concentration value in this ratiometric comparison skewed the data. Figure 6 represents the data obtained, which shows quencher **8** concentration on the x axis, against I_0/I. It is important to note that ten concentrations of both **1** and **5** were used and the line plots represent the average of the ten data series recorded.

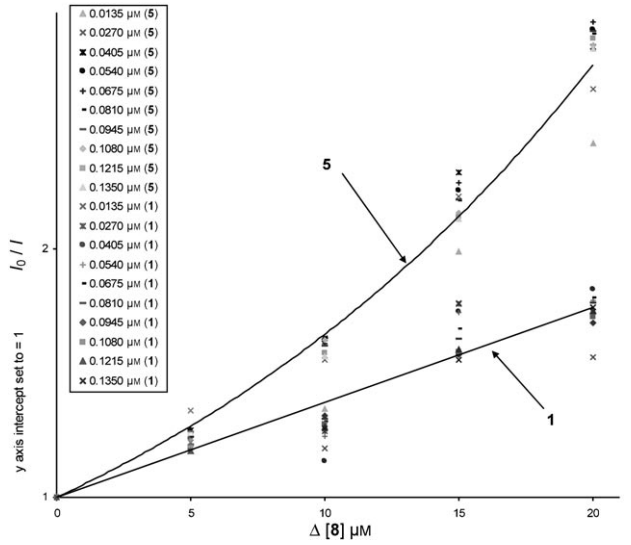
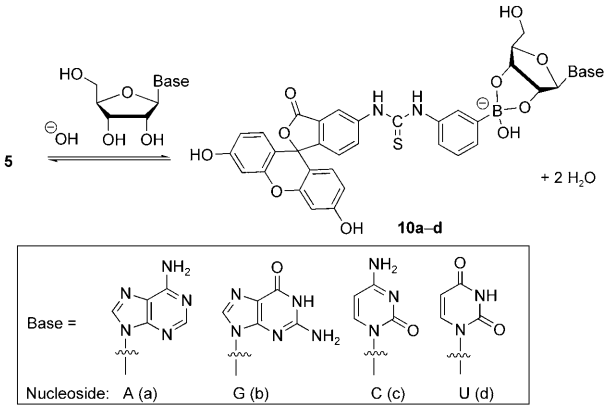


Figure 6. I_0/I plots for differing concentrations of **5** (upper) or **1** (lower) (0.0135 to 0.1350 μM) upon increasing concentration of quencher **8** [5 to 25 μM]. I_0 set at 5 μM **8**.

Figure 6 reveals a plot of I_0/I for increasing concentration of **8** on various concentrations of **1** and **5** (0.0135 to 0.1350 μM). The former, being essentially a linear relationship, confirms a dynamic quenching, whereas the upward concave curvature of the later relationship suggests both dynamic and static quenching are operating, which is in agreement with boronate ester formation.

Nucleosides as Diol Appended Quenchers

Nucleosides are the basic building blocks of RNA and DNA, and nucleoside analogues are a crucial part of acute myeloid leukaemia induction therapy, have been used to treat lymphoproliferative disorder, and have been used in tumour therapy. The effectiveness of nucleoside analogues as antiviral and antitumour agents has also provided impetus in the development of new approaches to their synthesis.[23–25] For example, the 2′,3′-didehydro-2′,3′-dideoxynucleosides (d4Ns) are a significant class of compounds active against the human immunodeficiency virus (HIV). In order to provide more data pertaining to the wider structure-activity relationship (SAR) a number of analogues have been synthesized and analyzed.[26–32] As such, nucleosides and their analogues represent important targets for the development of new sensing regimes.[33] Importantly, nucleosides have been previously shown to interact favorably with boronic acids through boronate ester formation *via* their 1,2-*cis*-diol motif.[34] Therefore, we set about probing the effect of adding nucleosides A (a), G (b), C (c) and U (d) to **5** to study the effect of covalently attaching *real life* quencher analytes to fluorescein derivatives through formation of boronate esters, general formula **10**, Scheme 6.



Scheme 6. Formation of **10 a–d** upon the addition of nucleosides adenosine A (a), guanosine G (b), cytidine C (c), and uridine U (d) to **5**.

Figure 7 shows a plot comparing the quenching ability of nucleosides on boronic acid appended quencher **5** as a ratio of enhancement over that with fluorescein **1**. Data were manipulated as per Figure 5. Since the nucleosides probed all present a diol motif in the same geometry, the propensity to form a boronate ester is the same in each case, and as expected, the data shown in Figure 5 displays no significant difference in the ability to form boronate esters. Thus, any intensity effect arising from more or less efficient energy transfer between different nucleosides is cancelled out in such a ratiometric comparison.

Although nucleoside G was a better quencher of both **1** and **5** (data not shown), the ratiometric comparison revealed that the enhanced quenching upon boronate ester formation

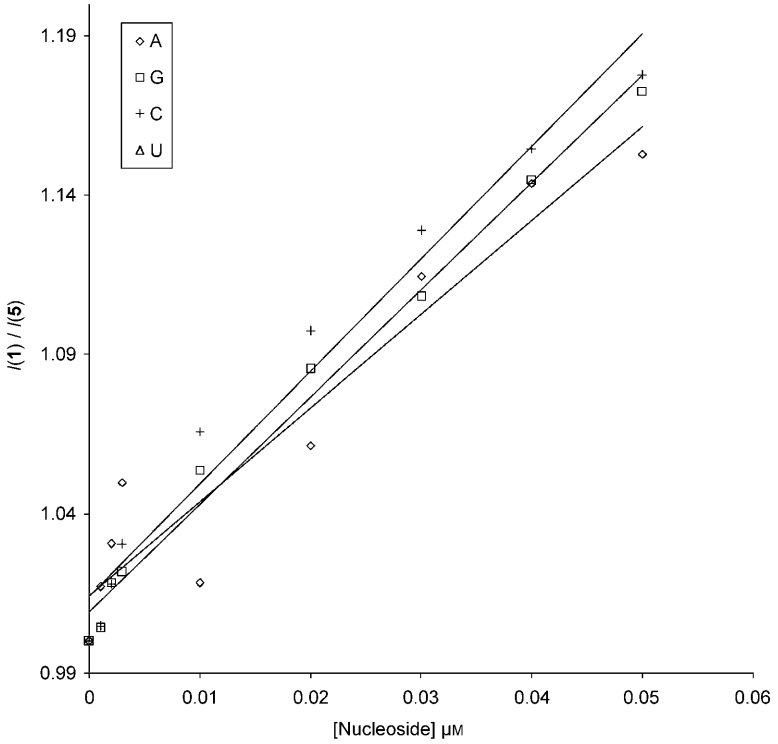


Figure 7. Plot ratiometrically comparing quenching ability of nucleosides on boronic acid appended quencher **5** as a percentage enhancement over that with fluorescein **1**.

is the same for A, G, C, and U, as judged by the essentially coincident plots obtained, Figure 7. This implies the binding strength of the nucleosides to the boronic acid of **5** is the same in each case, which one may expect since the 1,2-*cis*-diol binding motif is the same in each case. This demonstrates that such ratiometric comparison may be a viable estimator for relative binding constant information.

Conclusions

Three diol appended quenchers were synthesized and their interactions with a fluorescein derivative of a boronic acid, which was also synthesized, were probed. Boronate ester formation between diol and boronic acid was shown to enhance quenching by comparison with control systems. Methyl red was employed as a control quencher and fluorescein itself as a control fluorophore. That the quenchers synthesized had different absorption profiles and they elicited different responses with fluorescein appended boronic acid demonstrates the ready tuneability of this motif. The fluorescein boronic acid prepared was also shown to interact with nucleosides through boronate ester formation, and a common binding affinity for nucleosides was demonstrated.

Experimental Section

Reagents were used as commercially supplied by Sigma–Aldrich without further purification unless otherwise noted. 3-Aminobenzeneboronic acid was purchased from Frontier Scientific. Methanol refers to HPLC grade methanol. Fluorescence spectroscopy studies were conducted in pH 8.21 aqueous methanolic buffer solution, which consisted of 52.1 wt% HPLC grade methanol in deionised water with KCl (0.7456 M), KH_2PO_4 (0.3745 M), and Na_2HPO_4 (0.3914 M).[35]

A stock solution of fluorescein boronic acid (**5**) was prepared as follows: **5** (0.0527 g, 0.1 mmol) was dissolved in methanol (10 mL) and made up to 100 mL with aqueous methanolic buffer (pH 8.2). Then, 4.05 μL of the solution thus obtained was further diluted to 3 L with aqueous methanolic buffer to give 0.0135 μM solution. A fluorescein solution was similarly prepared.

Quencher stock solution preparation exemplified by **8**: A stock solution was prepared by dissolving **8** (0.0495 g, 0.1 mmol) in methanol (1.0 mL) made up to 10 mL with aqueous methanolic buffer (pH 8.2). Then 1.0 mL of this solution was made up to 10 mL with the aqueous methanolic buffer to give a 1 mM. Other quencher solutions were similarly prepared.

Nucleosides experiments: Fluorophores **1** and **5** were used at a concentration of 0.0675 μM, prepared by diluting 10 μL of 20 μM solutions in aqueous methanolic buffer to 3 mL. Nucleoside stock solutions of 125 mM were prepared also in aqueous methanolic buffer.

Instrumentation

Fluorescence spectroscopy measurements were performed using a Gilden Photonics FluoroSENS SENS-9000 instrument, with Starna Silica (quartz) cuvettes with 10 mm path lengths and four polished faces. Data was collected processed with the FluoroSENS 1.6 software package. Nuclear magnetic resonance spectroscopy measurements were performed using Bruker AVANCE 300 and 250 spectrometers in $CDCl_3$, CD_3OD, or CD_3OD+D_2O (2 drops). ^1H NMR spectra were recorded at 300 or 250 MHz, $^{13}C\{^1H\}$ at 75 or 63 MHz, and $^{11}B\{^1H\}$ at 96 MHz. Chemical shifts (δ) are expressed in parts per million and are reported relative to the residual solvent peak or tetramethylsilane as an internal standard and coupling constants (*J*) are given in Hertz.

Synthesis

3-(3-(3′,6′-Dihydroxy-3-oxo-3H-spiro[isobenzofuran-1,9′-xanthene]-5-yl)thioureido)phenylboronic acid (5): 3-Aminobenzeneboronic acid (**4**) (0.35 g, 2.57 mmol) was added to a solution of fluorescein isothiocyanate isomer I (**3**) (1.00 g, 2.57 mmol) in DMF (5 mL). The reaction mixture was stirred at room temperature for 12 h, then poured into methanol (10 mL). The solvents were removed in vacuo the residue was then dissolved in the minimum amount of fresh methanol. Chloroform was added and the product was obtained as a bright orange precipitate (920 mg, 68% yield). ^1H NMR (300 MHz, $CDCl_3$): $\delta_H = 7.97$ (1H, d, $J =$ 2.7), 7.74 (1H, dd, $J = 8.5$ and 2.7 Hz), 7.71 (1H, s), 7.6 (1H, d, $J =$ 8.5 Hz), 7.36 (1H, m), 7.19–7.15 (2H, m), 6.63–6.59 (5H, m), 3.35 ppm (2H, NH). $^{13}C\{^1H\}$ NMR (75 MHz, D_2O): $\delta_C = 182.2$, 179.9, 160.0, 159.9, 141.9, 141.8, 139.7, 133.1, 132.9, 132.1, 131.7, 131.5, 129.7, 128.2, 127.1,

126.6, 123.6, 123.4, 115.5, 114.9, 104.7, 104.5 ppm. $^{11}B\{^1H\}$ NMR (D_2O, 96 MHz): $\delta_B = 19.17$ ppm. HRMS (ESI)$^-$: *m/z* calcd for $C_{27}H_{18}BN_2O_7S$: 525.0933 $[M-H]^-$; found: 525.0946.

3-(Methyl(phenyl)amino)propane-1,2-diol (6): (±)Glycidol (9.3 mL, 140 mmol, 1.5 equiv) was added to a solution of *N*-methylaniline (10 g, 93 mmol) in methanol (100 mL). The mixture was heated to reflux for 48 h. On cooling the reaction mixture was concentrated in vacuo to give the desired product as colourless oil, which was deemed pure by inspection of the $^{13}C\{^1H\}$ NMR spectrum and no further purification was required. ^1H NMR (250 MHz, $CDCl_3$): $\delta_H = 7.19$ (2H, t, $J = 6.8$ Hz), 6.79–6.70 (3H, m), 3.97 (1H, ddd, $J = 12.0$, 4.3, and 4.3 Hz), 3.95–3.25 (4H, m), 2.95 ppm (3H, s). $^{13}C\{^1H\}$ NMR (63 MHz, $CDCl_3$): $\delta_C = 150.0$, 129.3, 117.5, 113.2, 103.8, 69.7, 64.3, 55.9, 39.4 ppm.

(*E*)-3-(Methyl(4-((4-nitrophenyl)diazenyl)phenyl)amino)propane-1,2-diol (7): To a stirred solution of 4-nitroaniline (1.00 g, 7.20 mmol) in a mixture of methanol, water, and aqueous hydrochloric acid (5 M) (1:1:1, 30 mL) at 0 °C, was added, dropwise, a chilled solution of sodium nitrate (0.2 M, 1.2 equiv, 8.7 mmol). Sulphamic acid was added to quench any remaining nitrite. After 30 min, a solution of **6** (1.25 g, 0.95 equiv, 6.9 mmol) in methanol and aqueous hydrochloric acid (1 M) (2:1, 30 mL) was added dropwise to the reaction mixture at 0 °C. Solid sodium acetate was added to the solution until pH 4 and allowed to stir overnight. The solution was extracted with dichloromethane (3×100 mL). The combined organic layers were dried over sodium sulphate and concentrated in vacuo to give the product as a dark red powder (89% yield, 2.02 g). ^1H NMR (300 MHz, $CDCl_3$): $\delta_H = 8.22$ (2H, d, $J = 9$ Hz), 7.82 (2H, d, $J = 9$ Hz), 7.79 (2H, d, $J = 9$ Hz), 6.73 (2H, d, $J = 9$ Hz), 3.92–3.85 (1H, m), 3.60–3.37 (4H, m), 3.02 ppm (3H, s). $^{13}C\{^1H\}$ NMR (75 MHz, $CDCl_3$): $\delta_C =$ 160.1, 157.2, 151.1, 148.0, 130.9, 129.0, 126.9, 115.9, 74.1, 68.3, 59.1, 44.0 ppm. HRMS (ESI)$^+$: *m/z* calcd for $C_{16}H_{18}N_4O_4$: 331.1406 $[M+H]^+$; found: 331.1399.

3-((4-((*E*)-(2,5-Dimethoxy-4-((*E*)-(4-nitrophenyl)diazenyl)phenyl)diazenyl)phenyl)(methyl)amino)propane-1,2-diol (8): To a stirred solution of 4-nitroaniline (5.00 g, 36 mmol) in a mixture of methanol, water, and aqueous hydrochloric acid (5 M) (1:1:1, 150 mL) at 0 °C, was added, dropwise, a chilled solution of sodium nitrate (0.2 M, 1.2 equiv, 43.5 mmol). Sulphamic acid was added to quench any remaining nitrite. After 30 min a solution of 2,5-dimethoxyaniline (5.28 g, 0.95 equiv, 34.5 mmol) in methanol and aqueous hydrochloric acid (1 M) (2:1, 150 mL) was added dropwise to the reaction mixture at 0 °C. Solid sodium acetate was added to the solution until pH 4 and allowed to stir overnight. The solution was extracted with dichloromethane (3× 200 mL). The combined organic layers were dried over sodium sulphate and concentrated in vacuo to give (*E*)-2,5-dimethoxy-4-((4-nitrophenyl)-diazenyl)aniline as a deep red powder (80% yield, 8.3 g). To a stirred solution of (*E*)-2,5-dimethoxy-4-((4-nitrophenyl)diazenyl)aniline (2.00 g, 6.6 mmol) in a mixture of methanol, water, and aqueous hydrochloric acid (5 M) (1:1:1, 30 mL) at 0 °C, was added, dropwise, a chilled solution of sodium nitrate (0.2 M, 1.2 equiv, 7.9 mmol). Sulphamic acid was added to quench any remaining nitrite. After 30 min a solution of **6** (1.14 g, 0.95 equiv, 6.3 mmol) in methanol and aqueous hydrochloric acid (1 M) (2:1, 30 mL) was added dropwise to the reaction mixture at 0 °C. Solid sodium acetate was added to the solution until pH 4 and allowed to stir overnight. The solution was extracted with dichloromethane (3× 100 mL). The combined organic layers were dried over sodium sulphate and concentrated in vacuo to give the product as a metallic appearance black/red powder (70% yield, 2.18 g). ^1H NMR (300 MHz, $CDCl_3$): $\delta_H =$ 8.30 (2H, d, $J = 9.1$ Hz) , 7.90 (2H, d, $J = 9.1$ Hz), 7.40 (1H, s), 7.26 (1H, d, $J = 7.2$ Hz) , 7.23 (1H, d, $J = 7.2$ Hz), 6.82–6.74 (2H, m), 6.35 (1H, s), 4.05–3.99 (1H, m), 3.98 (3H, s), 3.90 (3H, s), 3.78 (1H, dd, $J = 11.4$ and 3.3 Hz), 3.56 (1H, dd, $J = 11.4$ and 5.5 Hz) , 3.43 (1H, dd, $J = 14.5$ and 8 Hz), 3.30 (1H, dd, $J = 14.5$ and 5.5 Hz), 2.96 ppm (3H, s). $^3C\{^1H\}$ NMR (75 MHz, $CDCl_3$): $\delta_C = 157.7$, 156.7, 150.5, 147.5, 144.9, 142.4, 134.3, 129.7, 125.1, 123.0, 118.1, 113.7, 98.2, 97.8, 70.0, 64.7, 57.2, 56.4, 56.3, 39.8 ppm.

(*E*)-3-((4-((4-Chlorophenyl)diazenyl)phenyl)(methyl)amino)propane-1,2-diol (9): To a stirred solution of 4-chloroaniline (1.00 g, 7.84 mmol) in a mixture of methanol, water, and aqueous hydrochloric acid (5 M) (1:1:1, 45 mL) at 0 °C, was added, dropwise, a chilled solution of sodium nitrate (0.2 M, 1.2 equiv, 9.4 mmol). Sulphamic acid was added to quench any remaining nitrite. After 30 min a solution of **6** (1.35 g, 0.95 equiv, 7.5 mmol) in methanol and aqueous hydrochloric acid (1 M) (2:1, 45 mL) was added dropwise to the reaction mixture at 0 °C. Solid sodium acetate was added to the solution until pH 4 and allowed to stir overnight. The solution was extracted with dichloromethane (3×100 mL). The combined organic layers were dried over sodium sulphate and concentrated in vacuo to give the product as a yellow/orange powder (94% yield, 2.25 g). ^1H NMR (300 MHz, $CDCl_3$): $\delta_H = 7.78$ (2H, d, $J = 9.1$ Hz), 7.70 (2H, d, $J = 8.7$ Hz), 7.40 (2H, d, $J = 8.7$ Hz), 7.20–7.14 (2H, m), 3.02–3.92 (1H, m), 3.53–3.39 (4H, m), 3.05 ppm (3H, s). $^3C\{^1H\}$ NMR (75 MHz, $CDCl_3$): $\delta_C = 152.3$, 151.9, 150.5, 135.6, 129.8, 125.6, 119.5, 112.2, 70.3, 64.6, 55.4, 40.0 ppm.

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