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Selective fluorescence-based detection of dihydrouridine with boronic acids

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Abstract—The first fluorescent sensing system for dihydrouridine detection is presented. Dihydrouridine is the single most frequently occurring post-transcriptional modification in tRNA from bacteria and eukaryotes. A series of 10 boronic acid derivatives was prepared and their fluorogenic behaviours towards dihydrouridine and uridine were investigated. Whereas uridine always quenches fluorescence via π–π stacking interactions, several boronic acid sensors have been found to show substantial fluorescence enhancement upon binding with dihydrouridine.

The identification and characterization of ribonucleic acids (RNAs) and their post-transcriptional modifications are essential for fully understanding their structural and functional roles. These modifications are primarily found in stable RNAs such as transfer RNA (tRNA) and ribosomal RNA (rRNA). Roughly 100 different modified nucleosides have been isolated and characterized from tRNA.[1] After having performed their task, the tRNA molecules are cleaved to nucleosides. Whereas, unmodified nucleosides are recycled for biosynthesis of new RNA, modified nucleosides cannot be reutilized or further degraded but are either metabolized or excreted intact in urine. While healthy adults have stable baseline levels of modified nucleoside excretion, numerous works have shown that an elevated amount of modified nucleosides is related to carcinoma.[2]

The single most frequently occurring post-transcriptional modification in tRNA from bacteria and eukaryotes is dihydrouridine **D** in which the C5–C6 double bond of the uracil nucleobase is hydrogenated. Despite the widespread occurrence of dihydrouridine, little is known about its functional roles. It seems that dihydrouridine favors the C-2′-*endo* sugar conformation, which is less rigid than the C-3′-*endo* conformer, allowing conformational flexibility of RNA where tertiary interactions and loop formation must be simultaneously accommodated.[3]

The use of modified nucleosides excreted in urine as potential tumor markers has prompted the development of several analysis techniques. Traditionally urinary nucleosides are analyzed and quantified using HPLC analysis techniques. Most recently MALDI-TOF MS methods have been reported.[4] Because dihydrouridine, unlike all other natural or modified nucleosides, possesses no significant chromophore, HPLC analysis using UV detection is not practical due to poor sensitivity.[5] On the other hand, analysis using fluorescent sensors offers the advantages of being convenient and highly sensitive. Herein, we wish to report our efforts in developing the first fluorescent detection method of dihydrouridine.

An ideal fluorescent monitoring molecular system for specific recognition of nucleobases would display an enhanced signal upon binding. Unfortunately, fluorescence quenching by purine and pyrimidine nucleobases is a well known phenomenon which has been studied by various groups.[6] Usually, the nucleobases exhibit a variable degree of quenching, with guanosine being the most efficient, followed by adenosine, cytidine and thymidine–uridine. This has notably been used to monitor PCR,[7] to design probes for protein–ligand interaction[8] or for the design of a modified molecular beacon quenched by consecutive guanosines.[9]

Keywords: Fluorescence detection; Modified nucleosides; Tumor markers.
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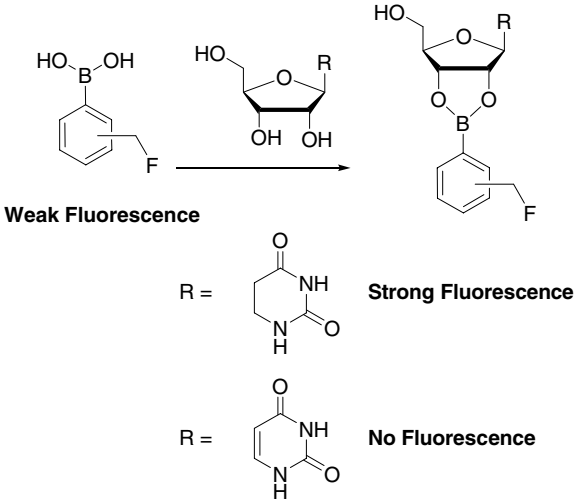
NMR and X-ray crystallography studies have shown that the nonplanar dihydrouridine resists stacking because of the absence of the C5–C6 double bond.[10] Therefore, we thought of taking advantage of the loss of aromaticity and thus of its quenching ability, upon saturation of the 5,6-double bond in dihydrouridine, for its specific fluorescence detection. This was done using boronic acids via formation of a boronate ester with the ribose 2,3-*cis*-diol group as depicted in Scheme 1.

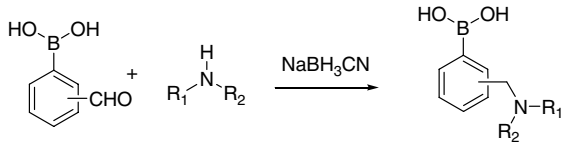
The design and synthesis of boronic acid-based fluorescent sensors for saccharide detection have been the subject of intense research in recent years.[11] There is general agreement that boronic acids covalently react with 1,2 or 1,3 diols to form reversible five- or six-membered cyclic esters. It has been demonstrated that these sensors show an increase in fluorescence intensity upon binding with a diol through different and still studied signalling mechanisms.[12] To date, and presumably, because of their quenching abilities, attempts to detect nucleosides or nucleotides with boronic acids have been made by using dye receptors,[13] polycations,[14] or molecular imprinting,[15] but never using fluorescent reporters. In this project, we chose to use a series of 10 fluorescent boronic acids which were synthesized according to reported procedures by reductive amination of readily available 2-, 3- or 4-formylbenzeneboronic acid (compounds **1a**–**c**, respectively), with the corresponding amine (Table 1). In this study, the sensors have been classified depending on their respective signalling mechanism, as ICT (internal charge transfer) fluorophore (Table 1, compounds **2a**–**f**), and PET (photoinduced electron transfer) fluorophores (Table 1, compounds **2g**–**2j**). In this latter case, it should be noted, that recent reports dispute the PET mechanism over a competing solvolysis process.[12]

The fluorescence titrations of sensors **2a**–**j** (2×10^{-5} M) were carried out in a pH 8.21 buffer (52.1% MeOH/H_2O phosphate buffer solution) in the presence of uridine or dihydrouridine (0–50 mM).[16] To our satisfaction, all candidates stood out showing modest to good fluorescence intensity enhancements upon addition of dihydrouridine. Fluorescence intensity enhancements induced by **D** and quenching efficiencies of **U** calculated from these titrations for sensors **2a**–**j** are given in Table 2. In the ICT series (Table 2, entries 1–6), there does not

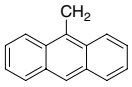
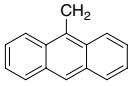
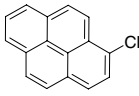
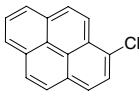


Scheme 1. Fluorescent response of boronic acids upon binding with uridine and dihydrouridine with different fluorophores F.

Table 1. Synthesis of fluorescent phenylboronic acid derivatives



1a-c **2a-j**

Entry	Starting material[a]	Product		Compound (Yield %)[b]
		R_1	R_2	
1	**1a**	Phenyl	H	**2a** (70)
2	**1b**	Phenyl	H	**2b** (62)
3	**1c**	Phenyl	H	**2c** (78)
4	**1a**	2-Fluorenyl	H	**2d** (80)
5	**1b**	2-Fluorenyl	H	**2e** (55)
6	**1c**	2-Fluorenyl	H	**2f** (53)
7	**1a**		H	**2g** (31)
8	**1a**		Me	**2h** (87)
9	**1a**		H	**2i** (25)
10	**1a**		Me	**2j** (80)

[a] Compounds **1a**–**c** refer to 2-, 3- or 4-formylbenzeneboronic acid, respectively.
[b] Isolated yields.

Table 2. Association constants (K_a) and fluorescence intensity changes of boronic acids with **D** and **U**

Entry	Sensor	K_a (M^{-1})[a]	ΔI_D[b] (fold)	Q_E[c] (%)
1	**2a**	64	1.5	86
2	**2b**	30	2	92
3	**2c**	162	5.5	82
4	**2d**	125	2.9	71
5	**2e**	146	2.8	54
6	**2f**	162	5.1	11
7	**2g**	383	1.39	21
8	**2h**	981	2.65	ND[d]
9	**2i**	224	1.6	83
10	**2j**	479	5.2	29

[a] Average of at least two measurements with $r^2 \geqslant 0.99$.
[b] Fluorescence intensity changes (I/I_0) upon addition of 50 mM of dihydrouridine **D**.
[c] Quenching efficiencies upon addition of 10 mM of uridine **U**.
[d] No quenching detected.

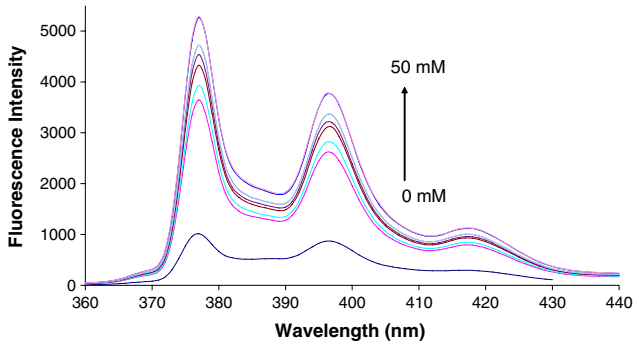


Figure 1. Fluorescence spectra change of **2j** (2×10^{-5} M) with different concentrations of dihydrouridine **D** (0–50 mM) in 52.1% MeOH pH 8.21 phosphate buffer. $\lambda_{ex} = 343$ nm.

seem to be any correlation to explain the differences observed in fluorescence intensity changes with the nature of the fluorophore. However, the *para* position appears to induce a superior emission intensity increase as shown by sensors **2c** and **2f** (5.5 and 5.1, respectively). In general, the fluorescence intensity increases are comparable with those obtained in the PET series. In this case, the largest intensity increase is observed with pyrene derivative **2j** (Table 2, entry 10). Addition of dihydrouridine at pH 8.21, induced a 5.2-fold intensity increase at 377 nm in a concentration-dependent manner (Fig. 1).

On the other hand, all sensors but one induced a gradual fluorescence intensity decrease upon addition of uridine (11–92% with 10 mM of **U,** see Supplementary data). In fact, the anthracene-based sensor **2h** (Table 2, entry 8), initially developed by the Shinkai group, binds strongly with **D**, but does not exhibit any important fluorescence change upon addition of 50 mM of **D**.[17] Most importantly, upon addition of **U**, the fluorescence intensity of sensor **2h** first augments and then diminishes slowly as the concentration of **U** increases. A high concentration of **U** (85 mM) is needed to recover the background fluorescence making this sensor inappropriate for dihydrouridine detection in the presence of uridine.

To examine the binding of sensors **2a**–**j** with **D** in a more quantitative fashion, the stability constants (K_a) were evaluated using the Benesi–Hildebrand equation for 1:1 stoichiometric binding (Table 2). In all cases a linear relationship is obtained when $1/(I - I_0)$ versus $1/([\mathbf{D}]_0)$ is plotted (see Supplementary data).[18] As it can be seen, the largest affinities of these various boronic sensors with dihydrouridine are obtained with PET sensors, **2h** and **2j** being the stronger ones ($K_a = 981$ and 479 M^{-1}, respectively). The solvation (size of the aromatic π-surface) and the steric crowding (number of *peri*-hydrogens of each fluorophore) need to be considered to interpret these values. Indeed, these binding constants are higher than those observed with phenylboronic acids and ribose.[19]

In order to specify the binding requirements, we compared the binding constants of compounds **2h** and **2j** with 2′-deoxydihydrouridine **dD**, and dihydrouridine 5′-monophosphate **DMP**. In **dD**, the 2′-OH group is missing and the boronic acid groups in **2h** and **2j** could theoretically complex the residual 3′,5′-dihydroxy group. On the other hand, in **DMP**, the 5′-OH position is phosphorylated and the 2,3-diol remains the sole complexation site. We found that the affinity of **2h** and **2j** with **dD** dropped severely compared to dihydrouridine. Whereas a low K_a of 11.6 M^{-1} was observed with **2j**, no spectral change was induced with **2h**, thus indicating the crucial role of the 2′-OH group in the complexation. In contrast, association constants of **2h** and **2j** obtained with **DMP** are, respectively, one half (297 M^{-1}) and one third (214 M^{-1}) of that for **D** presumably due to the enhanced hydrophilicity caused by the phosphate group.[13] These results clearly confirm the key role displayed by the 2,3-diol unit in the complexation.

Finally, the binding of **D** in the presence of **U** was assessed by a competitive fluorescence experiment. Sensor **2j** was choosen for this titration as it shows a 30% fluorescence quenching with 10 mM of **U**, a high affinity for **D** and one of the largest fluorescence intensity enhancement. The results show less sensitivity to **D** in the presence of **U** in agreement with the observed (quenched) complex formation with uridine. However, addition of a molar **D**/**U** ratio of 0.15 induced a gradual fluorescence increase indicating a high selectivity for **D** (Fig. 2). Moreover, with an equimolar amount of **D** and **U**, the fluorescence intensity increased up to almost 2 times the background fluorescence. The 5-fold fluorescence intensity increase observed with **D** is, however, never reached in the presence of **U**.

The present study has demonstrated the usefulness of boronic acid fluorescent sensors to detect the highly important dihydrouridine modified nucleoside. This was done by taking advantage of the reduction of the 5,6-double bond in uridine. Among the 10 sensors tested, five have been found to show significant fluorescence enhancement ($\Delta f > 5$). The transformation of the boronic acid function into a boronate ester selectively resulted in a pronounced OFF–ON-type fluorescent signaling behaviour. To the best of our knowledge, this is the first time that synthetic receptors have been used to fluorescently label a modified nucleobase. Moreover, these results revealed the sensitivity and selectivity of

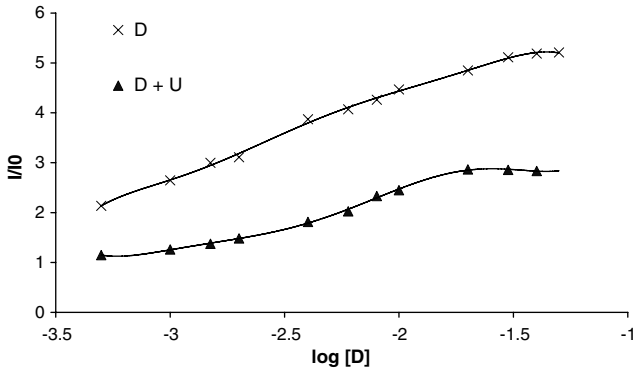


Figure 2. Fluorescence titration of boronic acid **2j** (2×10^{-5} M) with dihydrouridine in the presence of uridine (10 mM) in 52.1% MeOH pH 8.21 phosphate buffer.

these types of sensors allowing **2j** to probe small amount of **D** in a **U**/**D** mixture. These fluorescent reporter compounds might be very useful for subsequent application in the systematic study of the dihydrouridine urinary level in cancer patients.

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Supplementary data

Synthesis procedures and ^{1}H and ^{13}C NMR of **D**, **dD**, **DMP** and compounds **2a**–**j**. Experimental fluorescence measurements. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2006.10.150.

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