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Boronic acid-based fluorescent receptors for selective recognition of thymine glycol

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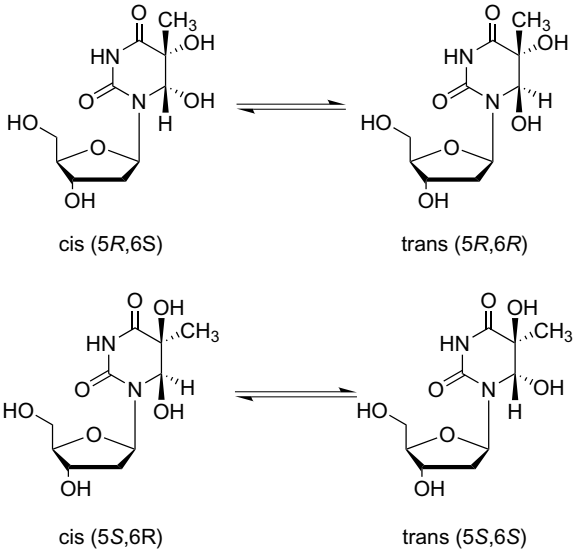
ABSTRACT

Thymine glycol is the major oxidation product of thymine. The amount of thymine glycol present within cellular DNA is one marker of the extent of oxidative damage, and chemosensors for thymine glycol have therefore a number of potential applications. In continuation of our studies devoted to the detection of modified nucleosides, we report herein our results toward the fluorescence detection of thymine glycol at both the nucleoside and the oligonucleotide levels using boronic acid sensors. These receptors show significant fluorescence enhancements and high selectivities in aqueous conditions upon binding with thymine glycol.

Reactive oxygen species (ROS) have been shown to generate several classes of damage to DNA.[1,2] As DNA, unlike proteins and lipids, cannot be replaced once modified, the role of ROS-induced DNA damage in the pathogenesis of many diseases has been highly emphasized.[3,4] Consequently, extensive studies have been undertaken to evaluate the contribution of oxidative DNA damage to aging, carcinogenesis, and other pathological processes. In this context, the development of analytical methods to investigate the presence and the role of oxidative DNA damage is of crucial importance. Among the 70 or so oxidized nucleosides which have been characterized to date,[5] thymine glycol (Tg, 5,6-dihydroxydihydrothymine) is the major stable product of thymine modification.[6] While it has been reported that thymine glycol is only slightly mutagenic,[7] it effectively blocks DNA replication and then may induce cell lethality.[8,9] In addition, as thymidine glycol (dThd-g) is excreted unaltered in the urine,[10–13] several methods have been developed for the measurement of this major base lesion in biological fluids and its use as a biomarker for oxidative DNA damage. Measurement of Tg lesions had been made using capillary-electrophoresis,[14] ^{32}P-postlabeling,[15] and HPLC-MS,[16,17] GC–MS[18] and MS/MS[19] tandem methods. Such assays aim at a better understanding of the biological significance of oxidatively generated DNA lesions in terms of mutagenic properties and repair processes.

In solution, thymidine glycol exists as two distinct diastereoisomeric pairs, namely (5*R*,6*S*; 5*R*,6*R*) and (5*S*,6*R*; 5*S*,6*S*), which are respectively in equilibrium with each other in favor of the cis-isomers (Scheme 1).[20] Interestingly, stereoselective excision of thymine glycol from oxidatively damaged DNA led researchers to



Scheme 1. Structures of the four 5,6-dihydroxy-5,6-dihydrothymidine isomers.

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highlight the importance of the cis stereoisomers over the trans forms.[21] Recently, we reported a fluorescence sensing system for dihydrouridine (DhU) detection, based on the formation of a boronate ester with the 2′,3′-cis-diol group of DhU.[22] While boronic acid-based fluorescent sensors have been used intensively for saccharides detection,[23] our study was the first to demonstrate the usefulness of such systems to detect a modified nucleoside. This selective fluorescence increase upon addition of dihydrouridine was done by taking advantage of the reduction of the 5,6-double bond in uridine which is otherwise a good fluorescence quencher. Herein, we wish to report an extension of our approach toward the fluorescence detection of Tg.

Although our final objective is the identification of Tg generated in vitro or in vivo, this study is dedicated to the detection of Tg prepared by chemical synthesis and purified by chromatography.[24] As it was the case with DhU, the loss of base aromaticity that accompanies the addition of hydroxyl groups at the C5–C6 positions of the ring in Tg should induce a fluorescence enhancement of the boronic probes upon formation of the boronic esters. cis-Diols are known to form strong reversible complexes with boronic acids,[25] and the use of a boronate-derived column has previously been shown to be an efficient alternative for *cis*-thymine glycol purification.[26] Our first set of experiments was therefore to identify a fluorescent boronic acid that would display an enhanced signal upon binding with Tg. Sensors **1–8** (Scheme 2) were therefore synthesized according to the published procedures,[27,22] and fluorescence titrations of **1–8** (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 Thymidine glycol (dThd-g) or Thymidine (T) (0–50 mM). Fluorescence intensity enhancements induced by dThd-g and the quenching efficiencies of T calculated from these titrations for sensors **1–8** are given in Table 1.

In all cases, the fluorescence intensity of these sensors augmented when increasing the dThd-g concentration. The highest fluorescence enhancements were obtained with sensors **3** (5.1 fold), **5** (4.1 fold), and **8** (2 fold). At the same time, all sensors

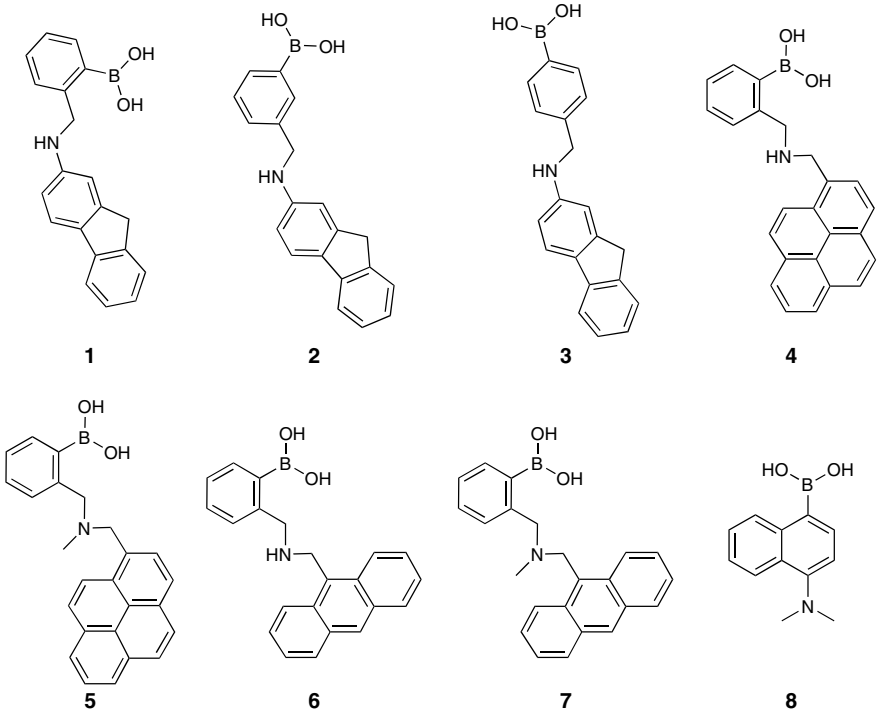
Table 1
Fluorescence intensity changes of sensors **1–8** in the presence of dThd-g and T

Sensor	λ_{ex} (nm)	λ_{em} (nm)	ΔI_D[a] (fold)	Q_E[b] (%)
1	301	386	1.1	88
2	294	384	2.3	81
3	303	383	5.1	84
4	343	377	1.4	52
5	343	377	4.1	20
6	388	414	1.2	0
7	388	414	2.5	0
8	300	445	2.0	96

[a] Fluorescence intensity changes (I/I_0) upon addition of 50 mM of dThd-g.
[b] Quenching efficiencies upon addition of 10 mM of T.

but the anthracenyl derivatives showed a gradual intensity decrease upon addition of T. These results indicate that the addition of dThd-g induces significant changes in the spectroscopic properties of these boronic acid-based sensors. The binding constraints of this association were further examined using a 3′,5′-di-*O*-acetyl-Thd-g. Fluorescence titration of sensor **5** (2×10^{-5} M) at different concentrations of this diacetylated dThd-g induced a 4.5-fold fluorescence increase in aqueous solution (52.1% MeOH/H_2O phosphate buffer, pH 8.21), thus demonstrating the crucial role of the C5–C6 dihydroxy functions.

We next evaluated the apparent association constants (K_a) between dThd-g (cis + trans forms) and the three sensors that gave the highest fluorescence enhancements assuming the formation of a 1:1 complex.[28] As exemplified in Figure 1 with sensor **5**, the apparent association constants were determined as 250, 300, and 55 M^{-1} for sensors **3**, **5,** and **8,** respectively. As HPLC measurement confirmed the presence of dThd-g as a mixture of diastereoisomers in a 70/30 cis/trans ratio, the K_a of **3**, **5,** and **8** therefore needed to be corrected in order to represent the affinity of the sensors for the cis-isomers. To confirm the hypothesis that the trans forms of dThd-g do not complex with boronic acids, we measured the fluorescence intensity change of sensor **5** (2×10^{-5} M) in anhydrous



Scheme 2. Fluorescent boronic acid derivatives studied.

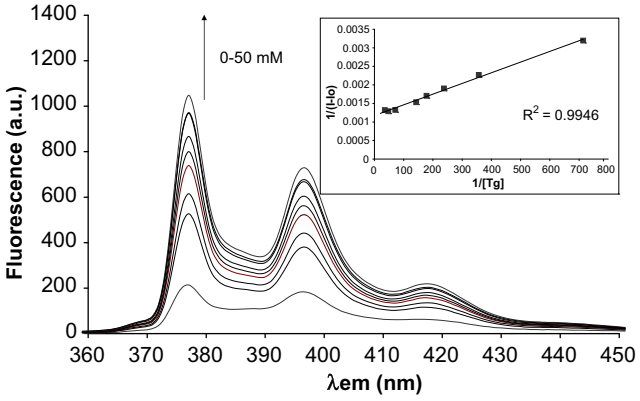


Figure 1. Fluorescence changes of sensor **5** (2×10^{-5} M) upon addition of dThd-g; inset : $1/(I - I_0)$ versus 1/[dThd-g] plot for determination of K_a ($400\ M^{-1}$).

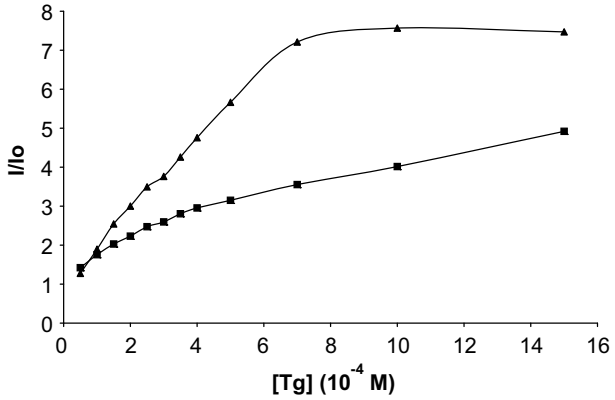


Figure 2. Fluorescence intensity changes of sensor **5** (2×10^{-5} M) upon addition of a 90/10 (▲) and 25/75 (■) cis/trans ratio of 3′,5′-di-*O*-acetyl-Tg in anhydrous DMSO.

DMSO upon addition of a synthetic 90/10 cis/trans ratio of the 3′,5′-di-*O*-acetyl-dThd-g. Under these conditions, a 7.5-fold fluorescence intensity increase was observed upon addition of 7×10^{-4} M of the diacetylated dThd-g (0.63 mM of the cis forms, Fig. 2). However, the addition of a synthetic 25/75 cis/trans ratio of the same compound induced a 3.2-fold fluorescence intensity increase which is attributed to the presence of 25% of the cis isomers. Indeed, the fluorescence intensity continued to increase and reached the 7.5-fold value upon addition of 2.7 mM of the 3′,5′-di-*O*-acetyl-dThd-g (0.67 mM of the cis forms). These observations dismiss the implication of the trans isomers in the fluorescence intensity enhancement observed. Based on these results, the K_a of sensors **3**, **5**, and **8** for *cis*-dThd-g were corrected as 333, 400, and 60 M^{-1}, respectively. Similarly, the corrected association constant of sensor **5** for the cis-isomers of the diacetylated analogue of Tg was determined as 2875 M^{-1}. This represents an improvement of about 7-fold in selectivity for the diacetylated compound. As both compounds adopt a favored anti conformation around the glycosyl bond, further studies are still needed to explain this selectivity. Nevertheless, these results further confirm the key role of the 5,6-dihydroxy ring.

Finally, the application of our method toward the detection of oligonucleotides containing Tg residues was evaluated. We anticipated fluorescence quenching of the fluorophore by proximal bases upon boronate formation. Indeed, it is well documented that the fluorescence-quenching phenomenon induced by nucleobases takes place through a photoinduced electron transfer mechanism and is highly dependent on both the chromophores and the

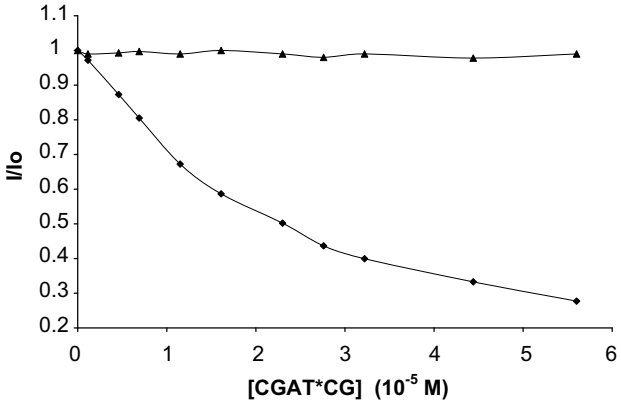


Figure. 3. Fluorescence intensity changes of sensor **5** (2×10^{-5} M) upon addition of d(CGATCG) (▲) and d(CGATgCG) (■) in a pH 8.21 phosphate buffered solution at 30 °C.

distance between them.[29,30] We therefore needed to evaluate the specificity of this quenching event. Following treatment of d(CGATCG) in a 2% OsO_4 aqueous solution, we were able to isolate a single Tg-containing oligonucleotide sequence by HPLC. Titration experiments were performed by adding increasing amounts of the oxidized sequence to a buffered solution of **5** (2×10^{-5} M, in PBS buffer, pH 8.21). To avoid the formation of secondary structures and to allow a better comparison with nondamaged DNA, these titrations were done beyond the melting temperature of d(CGATCG). In fact, denaturation experiments showed that the oxidized sequence does not self-assemble at all (data not shown). Figure 3 shows that the fluorescence of **5** is effectively quenched by the addition of the Tg-containing sequence. In a marked contrast, no fluorescence quenching was observed with the addition of the unmodified sequence d(CGATCG) under the same conditions. This demonstrates the high selectivity of boronic acid sensors toward DNA sequences having Tg residues. Data collected from this titration were used to generate a Stern-Volmer plot in the concentration range of $0–6 \times 10^{-5}$ M and were found to fit the static quenching model giving a quenching constant of $5 \times 10^4\ M^{-1}$.[31] The formation of a boronic ester was further confirmed by adding 50 equiv of fructose to the final solution. This addition led to the reversal of the quenching phenomenon in a concentration-dependent manner indicative of a competition between the two diol systems (see Supplementary data). Hence, these results revealed the importance of these Tg-selective functional receptors for the discriminatory detection of oxidized DNA sequences in aqueous conditions.

In conclusion, the present study demonstrated the ability of boronic acid fluorescent sensors to detect specifically the cis-stereoisomers of Tg at both the nucleoside and the oligonucleotide levels. Considering the importance of thymine glycol lesions in ROS-induced DNA damages, the discovery of Tg-selective fluorescent sensors will aid the effort of developing new sensitive oxidized DNA lesion-sensing analytical devices.

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Supplementary data

Fluorescence measurements and *K*a determination. Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.tetlet.2008.07.173.

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