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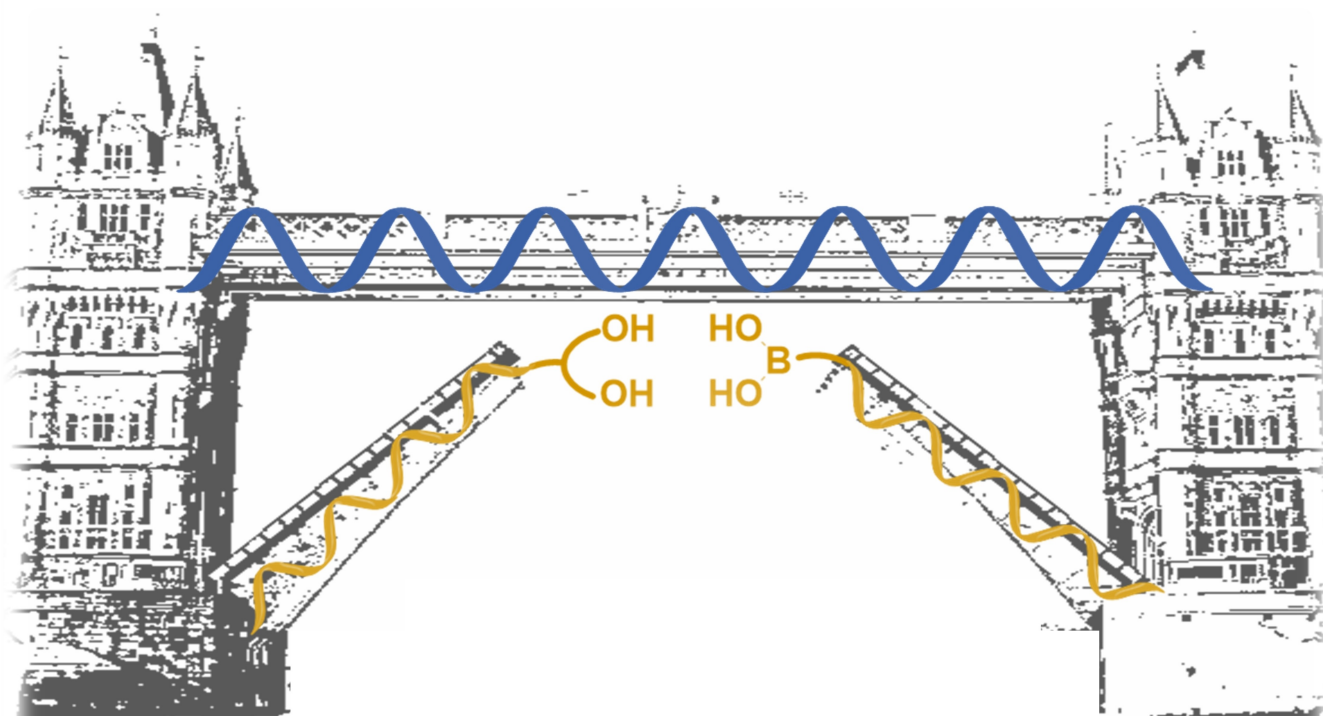
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Applications of the Reversible Boronic Acids/Boronate Switch to Nucleic Acids

Mégane Debiais, Jean-Jacques Vasseur, and Michael Smietana^{*[a]}



Abstract: Over the last decades, boron and nucleic acids chemistries have gained a lot of attention for biological, medicinal and analytical applications. Our laboratory has a long-standing interest in both chemistries and owing to the ability of boronic acids to react with *cis*-diol function in aqueous media we developed over the years a variety of applications ranging from molecular recognition and sensing to the development of reversible dynamic systems in which the natural phosphodiester linkage was replaced by a boronate. In this account, we summarize research results from our group from our preliminary studies on molecular recognition of ribonucleosides to the dynamic assembly of functional DNazymes. In particular, the various parameters influencing the dynamic nature of these reversible covalent bonds able to respond to external stimuli are discussed. Finally, current challenges and opportunities for boron-based nucleic acids are also addressed.

Keywords: nucleic acids, boronic acids, self-assembly, templated ligation, conjugation

1. Introduction

The boron atom, first discovered by French Chemists Gay-Lussac and Thenard,^[1] is the fifth element of the periodic table and has three valence electrons (electronic configuration $1s^2 2s^2 2p^1$). Pure boron is very rare on Earth and exists mainly as minerals containing boron oxide in various proportions, the most known forms being certainly tincal, the impure form of borax ($\text{Na}_2\text{B}_4\text{O}_7 \cdot 10\text{H}_2\text{O}$) and sassolite (H_3BO_3). While natural borate complexes are produced by microorganisms and algae, naturally occurring organoboron compounds remains unknown to date.^[2,3] However, a large variety of methods have been described to synthesize compounds containing carbon-boron bonds.^[4,5] Among these compounds, boronic acids most commonly found in their neutral trivalent form in which the boron atom has a sp^2 geometry with a vacant p orbital and a trigonal planar geometry are of particular interest.^[6] Because of their ability to bind reversibly with 1,2- and 1,3-diols, boronic acids have found wide applications as saccharides sensors.^[7] While boronate esters are classically formed in anhydrous organic medium by dehydration, boronic acids and their boronate esters are in equilibrium in aqueous medium (Figure 1a). Disfavoured at low pH, addition of hydroxyl anions shifts the equilibrium toward the formation of a hydroxy boronate ester by complexation of the vacant p orbital of the boron atom. As a consequence of the release of the angle strain around the boron atom, from 120° to 109° for the O–B–O and B–O–C angles, a resulting negatively charged

tetravalent compound with a tetrahedral sp^3 geometry is generated.^[8–10]

The formation of boronate esters could also be used to protect boronic acids against oxidation reactions.^[8] Several diols are used for this purpose, the most common being pinacol and catechol. The trivalent ligand *N*-methyliminodiacetic acid (MIDA), recently developed by Burke and coworkers, is also widely used for its ability to stabilize boronate esters through the complexation of the vacant p orbital of the boron atom by the nitrogen atom (Figure 1b).^[11,12]

Because of their structural and electronic properties, the attention paid to organoboron derivatives and particularly boronic acids has considerably increased over the past 20 years. These compounds have found numerous applications in organic synthesis and in particular in cross-coupling reactions. The tremendous work made by Akira Suzuki for the development of palladium-catalyzed cross coupling of boronic acids has been rewarded with the 2010 Nobel Prize in Chemistry along with Ei-ichi Negishi and Richard Heck.^[4,6,13] Boronic acids and their analogues are also widely used as chiral auxiliaries in asymmetric synthesis,^[14] as catalysts,^[15,16] as chemotype in medicinal chemistry,^[17,18] in molecular recognition^[19], for the formation of bioconjugates^[20,21] and for the development of supramolecular and self-healing materials.^[22–26]

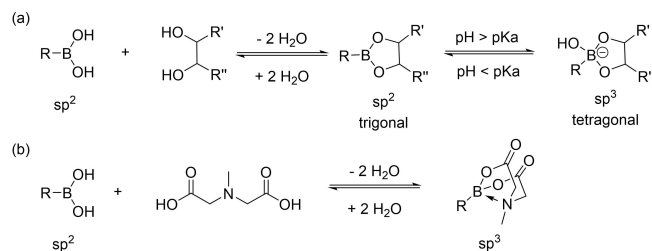


Figure 1. (a) General scheme of the formation of a boronate ester and (b) formation of a MIDA-boronate ester by dehydration

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Many of these applications rely on the interaction of boronic acids with alcohols, in particular *cis*-1,2- and *cis*-1,3-diols, to control the geometry of the assembly.^[27] Therefore, during the past several decades boronic acids were widely used in the development of probes for the detection of saccharides, polysaccharides or glycoproteins.^[7,28,29] Moreover, the binding strength of boronic acids to saccharides is determined by the orientation and relative position of the hydroxyl groups, thus allowing a structural differentiation between analogous saccharidic molecules. Boronic acid-based polymers were also used since the 70s for the separation of mixtures of deoxy- and ribonucleosides.^[30,31]

In this context, more than 15 years ago we initiated a research project aimed at combining the specific properties of boronic acids with those of nucleic acids, exploiting in particular the diol function of ribonucleosides which thus appeared as a formidable recognition element. This journey led us to consider the boronate function as a mimic of internucleoside linkages and eventually to use these properties for the control of functional architectures. Our research accomplishments over the years are presented in this personal account.



Mégane Debiais received her PhD in 2020 from the University of Montpellier working on developing new programmable boronic acid-based split DNAszymes for molecular recognition under the guidance of Prof. Michael Smietana. She focused also on boronic acid-based material assembly. She is currently a Postdoctoral fellow under the joined supervision of Prof. Michael Smietana (Institute of Biomolecules Max Mousseron) and Prof. Jean-Marc Campagne (Institute Charles Gerhardt of Montpellier) exploring the development of “DNA-encoded libraries”.



Jean-Jacques Vasseur received his Ph.D. in 1988 from the University of Montpellier working on the reactivity of DNA apurinic sites under the guidance of Prof. Jean-Louis Imbach and Dr Bernard Rayner. From 1990 to 1992, he was a visiting scientist at IONIS Pharmaceuticals in Carlsbad, CA (USA) where he developed oligonucleotide analogues without phosphorus backbone. In 1993, he returned to Montpellier and was promoted CNRS Research Director in 1998. He is currently vice-director of the Max Mousseron Institute of Biomolecules (IBMM) in Montpellier. In 2020, he was elected vice-president of the International Society of Nucleosides, Nucleotides and Nucleic Acids (IS3NA). He co-authored more than 250 publications on various aspects of nucleic acid chemistry focusing notably on the design of chemically modified DNAs and RNAs and their conjugates for therapeutic and diagnostic applications.



Michael Smietana was educated at the Ecole Normale Supérieure in Paris. He received his PhD in 2001 from the University of Strasbourg under the guidance of the late Dr Charles Mioskowski. He then joined Stanford University as a Postdoctoral Fellow in Prof. Eric Kool's group for two years. In 2004, he moved to the University of Montpellier as an Assistant Professor, working in the Nucleic Acids Department of the Institute of Biomolecules Max Mousseron (IBMM). He was appointed Associate Professor in 2010 and Full Professor in 2012. In 2018 he received the Jean-Marie Lehn award from the Organic Chemistry Division of the French Chemical Society. Since 2020 he leads the ChemBioNAC team at Institute of Biomolecules Max Mousseron. His current research interests focus on molecular recognition, DNA-based asymmetric catalysis and nucleic acid bioconjugates.

2. Molecular Recognition

2.1. Fluorescent Sensors

While the use of fluorescent probes exploiting the boronic acid/diol pair for the recognition of saccharides has been the subject of numerous works,^[7,28,29] the detection of nucleosides or nucleotides by direct fluorescence emission has not been much discussed in the literature in large part because of the fluorescence quenching capacity of purines and pyrimidines.^[32]

We have therefore started our work by studying the ability of fluorescent boronic probes to detect modified nucleosides. The identification and characterization of nucleic acids involved in transcription and translation processes, and in particular nucleic acids resulting from post-transcriptional modifications, are essential to understand their functional role and to detect genetic mutations. While unmodified nucleosides are recycled for the biosynthesis of new nucleic acids, modified nucleosides are usually metabolized or excreted intact in the urine. More than a hundred modified nucleosides have already been characterized from transfer RNA^[33] and significant over-expression of these modified nucleosides traditionally analyzed and quantified by HPLC,^[34] capillary electrophoresis^[35] or mass spectrometry^[36] may in some cases reveal the presence of carcinomas. Despite considerable progress in the detection and quantification of these species, classical analytical techniques are difficultly applicable to modified nucleosides lacking a chromophore moiety. Among these compounds, we were first interested in dihydrouridine **1** which is the most frequently observed post-transcriptional modification in bacterial and eukaryotic transfer RNAs and also found in large quantities in the urine of cancer patients.^[37] In dihydrouridine the C₅-C₆ double bond of the uracil nucleobase is hydrogenated which leads to a loss of aromaticity and thus of absorbance. Fluorescence detection of natural nucleosides is generally difficult because of the extinction capacities of purines and pyrimidines. However, in the case of dihydrouridine **1**, the loss of aromaticity leads to a strong decrease of its quenching capacity. We therefore considered developing boronic acid-based fluorescent sensors capable of detecting dihydrouridine with good sensitivity (Figure 2).^[38]

Inspired by sensors developed for saccharides detection,^[39] we synthesized a series of 10 fluorescent boronic acids that were obtained by reductive amination of 2-, 3- or 4-formylbenzeneboronic acid **3a–c** with various amines (Figure 3).^[38] In presence of dihydrouridine modest to good increase of fluorescence intensities were observed for all sensors but the best results were obtained with fluorophores **4c**, **4f** and **4j** which had fluorescence intensity changes of a factor of 5.5, 5.1 and 5.2 respectively. In contrast, in the presence of uridine, a decrease in fluorescence intensity was observed for all compounds to varying degrees (0 to 92%). Furthermore, no change of fluorescence emission was observed with 2'-

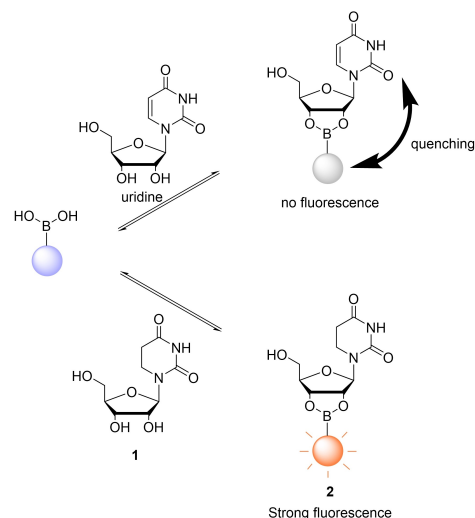


Figure 2. Schematic representation of fluorescent detection of dihydrouridine **1** by a boronic acid-based sensor.

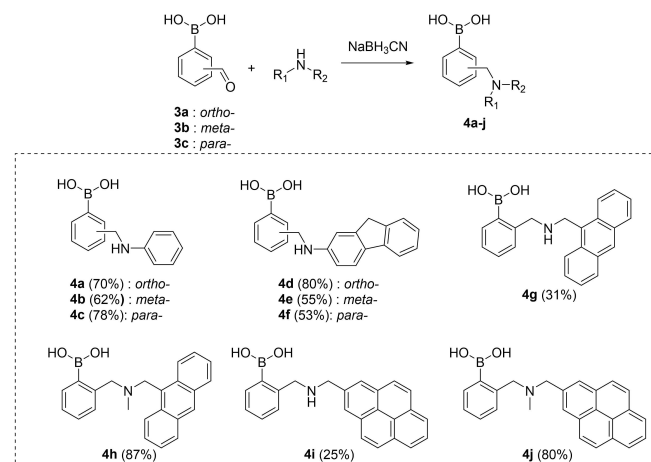


Figure 3. Fluorescent boronic acid-based sensors evaluated for dihydrouridine detection.

deoxydihydrouridine highlighting the crucial role of the 2',3'-diol group in the complexation. Finally, competitive fluorescence experiments were then performed and showed that dihydrouridine could be detected in a uridine/dihydrouridine mixture. These results showed for the first time the usefulness of boronic acids for the fluorescence detection of modified ribonucleosides and encouraged us to continue our research.

This concept was then applied to *cis*-thymidine glycol **5**, another modified nucleoside produced during the oxidation of thymine by reactive oxygen species.^[40] Slightly mutagenic, thymidine glycol blocks DNA replication and may induce cell lethality.^[41] Based on the results obtained with

dihydrouridine,^[38] boronic acid-based sensors **4d–j** and **7** (Figure 3 and Figure 4a) were evaluated in presence of *cis*-thymidine glycol and all of them led to fluorescent enhancements. Best results were obtained with sensors **4f**, **4j** and **7** which displayed fluorescence intensity changes of a factor of 5.1, 4.1 and 2 respectively. We then studied the possibility of detecting thymidine glycol within oligonucleotide sequences. For this purpose, the deoxy-oligonucleotide d(CGATCG) was treated with an aqueous solution of osmium tetroxide in order to generate the thymidine glycol residue (Figure 4b). In the presence of this modified oligonucleotide, a specific fluorescence quenching was observed due to the quenching ability of the proximal nucleobases. In contrast, no residual fluorescence change was observed in the presence of the

unmodified oligonucleotide due to the absence of any diol unit.

Boronic acid-based fluorescent sensors thus represent a promising way to develop sensitive analytical devices for the detection of specific DNA lesions.

2.2. Molecular Recognition of Modified Nucleosides Studied by NMR

In order to enhance the interaction between boronic acids and nucleoside derivatives, we introduced iminodiacetic acid (MIDA) or iminodiethanol (DEA) functions in the 3'-position of a thymidine derivative (Figure 5).^[42] Unlike traditional boronate esters that are stabilized at high pH, MIDA boronates are hydrolysed in basic aqueous solution and DEA boronates are deprotected in acidic aqueous solution.^[11] The formation of boronate esters between these compounds and phenyl- or butylboronic acid was then investigated by NMR in anhydrous DMSO-*d*₆. A 1:1 mixture of **11** and butylboronic acid analysed by ¹H NMR, led to splitting of H₃, H₆ and H₁ protons and appearance of a new set of peaks around 4.0–4.3 ppm characteristic of the formation of a boronate ester. These results were confirmed by ¹³C NMR in which a shifting of the *N*-methylene and boron-bound carboxylic acid carbons was observed. The high shift signal from 32.5 to 11.2 ppm on the ¹¹B NMR also indicated a tetrahedral environment of the boron atom induced by the dative B–N bond. Similar observations were made when **12** was mixed with phenyl- or butylboronic acid. However, 2D NMR analysis revealed that the flexible boronate formed between **12** and boronic acids undergoes a ring opening-closure equilibrium associated to the breaking of the B–N bond while in the case of **11** rather rigid structures were obtained.

This study confirmed the ability of boronic acids to generate boronate esters with nucleosides containing iminodiacetic acid or iminodiethanol functions. This prompted us to evaluate the possibility of replacing the natural phosphodiester linkage present in DNA and RNA by a boronate one that could be generated through the ligation of two modified oligonucleotide strands, one comprising a ribonucleoside at the 3'-extremity while the second one would be modified by a boronic acid at the 5'-extremity.

3. DNA-Templated Chemical Ligation

3.1. Synthesis of Boronucleotides and Formation of Dinucleotides

First, molecular modeling was initiated on modified nucleotides carrying a 5'-boronic acid in order to evaluate the deviation from the structural and electronic shapes of the natural nucleotides monophosphates.^[43] The structure of these

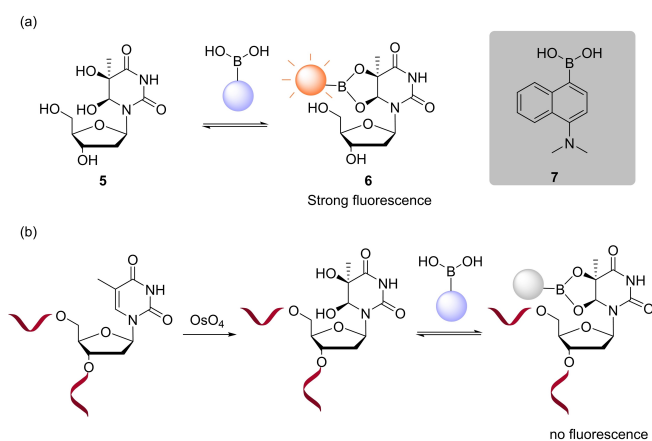


Figure 4. Fluorescent detection of *cis*-thymidine glycol **5** at the (a) nucleoside and (b) oligonucleotide levels.

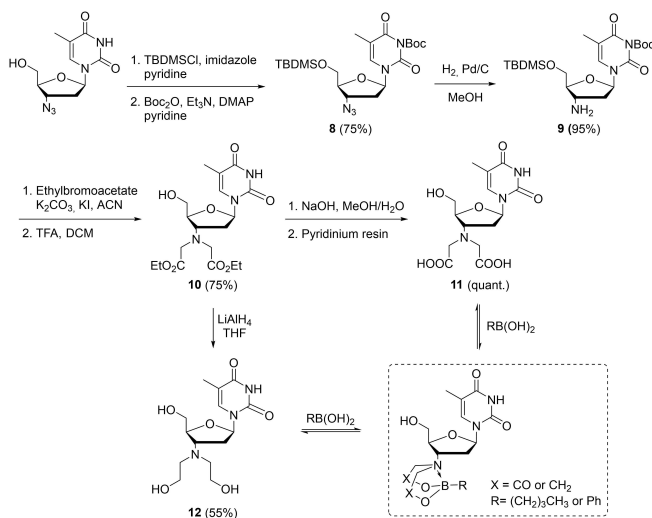


Figure 5. Synthesis of the thymidine analogues **11** and **12** and their associations with boronic acids.

analogues that we named boronucleotides (**17**, **18**, **19** and **23**, Figure 6) were optimized and their mapped electrostatic potentials showed that their electrostatic charges were similar to natural nucleotides monophosphates thus indicating that Watson-Crick hydrogen bonding would not be affected by the presence of a boronic acid group. Structural fits and bond measurements were then performed between optimized structures and their monophosphate counterparts. Here again, no strong structural variation was observed. The maximum variation observed for the C₄-oxygen of the acid function distance was about 0.1 Å. Boronucleotides thus represent a nearly perfect mimic of the natural nucleotides monophosphate. We therefore embarked on the synthesis of boronic acid analogues of 5'-nucleotide monophosphate starting from commercially available 5'-dimethoxytrityl-nucleosides (Figure 6).^[44] After a series of protection and deprotection steps, the 5'-hydroxy group was oxidized with DMSO and dicyclohexylcarbodiimide (DCC) in the presence of dichloroacetic acid in leading to the corresponding aldehyde **14**. A homologation step was then performed in presence of dimethyl-1-diazo-2-oxopropylphosphonate (Bestmann-Ohira reagent) to furnish alkyne **15**. After semi-hydrogenation of the alkyne, a borylation of the resulting alkene using diisopinocampheylborane yielded the corresponding 3'-OTBDMS-boronic acid. Deprotection of the 3'-hydroxyl function under aqueous acidic conditions led to the expected boronucleotides. Overall, the synthesis of boronucleotides **17**, boronucleotides **18** and

boronucleotides **19** necessitated 7 steps for an overall yield of 31%, 38% and 28% respectively.^[43,44] In the case of the analogous boronucleotide **23**, the boron atom was introduced *via* a metathesis with vinyl 2-methyl-2,4-pentanediol boronate in presence of Grubbs-Hoveyda II catalyst. The boronucleotide **23** was obtained in 9 steps with an overall yield of 25% after deprotection and reduction of the double bond.^[43] Over the years, synthetic improvements were made and we routinely perform now the oxidation step with 2-iodoxybenzoic acid (IBX) while the homologation reaction is now directly leading to the alkyne using either the Tebbe (bis(cyclopentadienyl)- μ -chloro(dimethylaluminum)- μ -methylene-titanium) or the Nysted (cyclo-Dibromodi- μ -methylene- μ -(tetrahydrofuran)]trizinc) reagent.

The ability of boronucleotides to form a boronate junction with uridine leading to the formation of dinucleotides was first studied by ¹H NMR in anhydrous DMSO-d₆. All of them were found to bound to uridine and significant changes in the exchangeable protons were observed. Indeed, the 2'- and 3'-hydroxyl as well as free hydroxyl protons carried by boron disappeared. We also demonstrated that the boronate internucleosidic linkage could be formed in aqueous basic medium. The sugar conformation of the formed structures was then investigated using the theory of Altona^[45,46] and Obika.^[47] Usually, free uridine is in equilibrium between the C_{2'}-endo (South) and C_{3'}-endo (North) conformations with a slight prevalence for the C_{2'}-endo conformation but in presence of the boronucleotide a lower coupling constant value was observed thus indicating a change of the ring pucker which appeared to adopt about 75% of the North conformation. The binding affinity of **17** for different ribonucleosides monophosphates evaluated in aqueous solution using Springssteen and Wang's colorimetric assay^[48] revealed a higher binding constant for CMP (324 M⁻¹) followed by UMP (235 M⁻¹), GMP (215 M⁻¹) and AMP (120 M⁻¹). Considering that pyrimidine nucleosides adopt preferentially a North-like conformation due to stronger anomeric effects, the formation of this reversible boronate internucleosidic linkage seemed to be dependent on the preorganized North-like sugar conformation of the diol moiety.

This study demonstrated the formation of a dinucleotide between a boronucleotide and uridine and opened the way for the development of dynamic and reversible DNA-templated ligation through the reaction of two modified oligonucleotides, one containing the boronucleotide residue **17** at its 5'-extremity and the other a ribonucleotide at its 3'-extremity.

3.2. DNA-Templated Ligation

The preparation of an oligonucleotide modified by a boronic acid function at its 5'-end requires the incorporation by

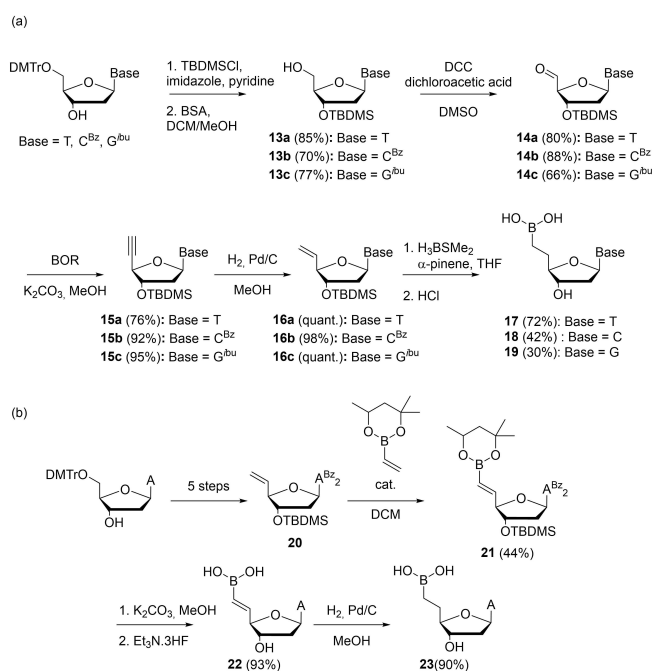


Figure 6. Synthesis of Boronucleotide derivatives of (a) T, C and G and (b) A. (BOR : Bestmann-Ohira reagent)

automated synthesis of a boronucleotide bearing a phosphoramidite group at the 3'-position (Figure 7).^[49] We decided to incorporate the thymidine analogue whose boronic acid function had to be protected before the phosphitylation step. Initially used in the form of a pinacol ester, we now opt for the more stable MIDA group, whose deprotection in basic medium is concomitant with the cleavage of the support. With compound **25** in hand we synthesized a T_6T^{bn} sequence that was designed to reversibly ligate with a second strand carrying a ribonucleotide at its 3'-end in presence of a DNA-temple.

The ability of the 14-mer template to bring the two oligonucleotides in close proximity and promote the ligation was evaluated by UV thermal denaturation in the presence of various stimuli.^[49,50] A control experiment was first performed at pH 7.5 in presence of the 14-mer template, a non-modified T_7 sequence and the sequence 5'-GCGTCGrC-3' carrying the required *cis*-diol function at its 3'-end. This led as expected to a double sigmoid transition corresponding to the two half-duplexes and a T_m value of 12.3 °C for the less stable half-duplex. In contrast, when T_7 was replaced by T_6T^{bn} a stabilization of 6.8 °C ($T_m = 19.1$ °C) was observed at pH 7.5 (Figure 8). To confirm that the stabilization was consequent to the formation of a boronate internucleoside linkage, 5'-GCGTCGrC-3' was replaced by an analogous sequence having a 3'-deoxycytidine end. In that case, a T_m of 12.8 °C value similar to the one observed with T_7 confirmed the crucial role of the 3'-end ribonucleotide for the formation of the modified internucleoside linkage.

The formation of boronate esters being pH-dependent and highly promoted in aqueous basic medium, the pH value was thus increased from pH 7.5 to 9.5. This led to an impressive

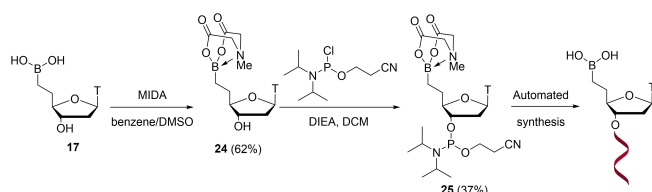


Figure 7. Incorporation of the boronucleotide into oligonucleotide sequences.

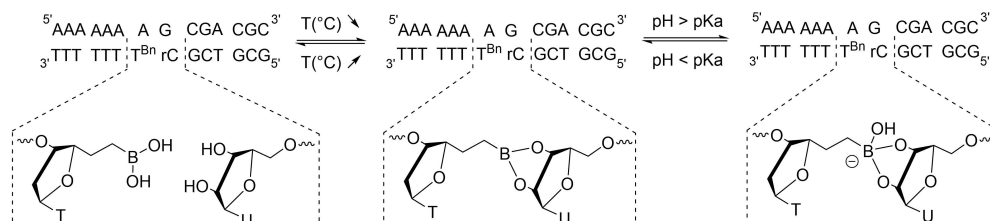


Figure 8. Dynamic and reversible DNA-templated ligation.

stabilization of 14.4 °C ($T_m = 26.7$ °C) and confirmed the formation of a negatively charged hydroxyboronate internucleoside linkage consecutive to the transition of the hybridization state of the boron atom from sp^2 to sp^3 . While these results demonstrated for the first time how boronic acid could be used to promote self-organization and ligation of strands in enzymatic-free conditions, it should however be noted that the boronate junction is destabilizing compared to the corresponding non-modified 14-mer duplex. A competition experiment made in the presence of an excess of fructose confirmed that once dissociated the system was no longer able to reassociate.

Interestingly, when a RNA template is used instead of the DNA template the melting temperature is 14.0 °C higher at pH 7.5 but less pH-dependent thus suggesting that the boronate junction might favour an A-like over a B-like helical conformation.^[43] This first set of results opened the possibility to generate biologically relevant dynamic systems that may find useful applications notably in the preparation of biocompatible materials.

3.3. Influence of the Nature of the 3'-Extremity for DNA-Templated Ligation

In order to examine the influence of the nature of the diol at the 3'-end we synthesized a series of ribonucleotide strands modified at their 3'-end by different dihydroxy moieties (Figure 9).^[51] Thus, the acyclic derivative **26** and the 2'-methyl analog **27** allow to evaluate the influence of flexibility or additional constraint on the sugar conformation. The possibility of generating oxazaborolidine instead of boronate linkages was evaluated with aminonucleosides **28** and **29**. Finally, the influence of the base was evaluated with the abasic derivative **30** and in the case where an additional *cis*-diol unit **31** is added at the 3'-end. Examination of the thermal denaturation curves of these different systems in the presence of the T_6T^{bn} sequence and a 14-mer template revealed that only the 2'-methyl modification was able to generate linkages whose stability increased between pH 7.5 and 9.5 (from $T_m = 11.0$ to 16.1 °C). These values were still much lower than those obtained with uridine ($T_m = 20.9$ to 30.8 °C). All other modifications resulted in destabilization compared to their

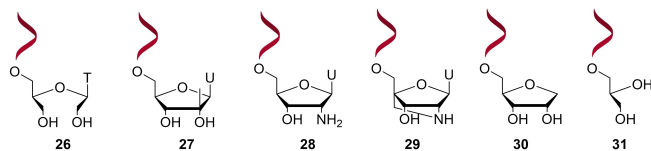


Figure 9. Structures of 3'-end modified ribonucleosides.

unmodified analogues and no dependence on pH variations, thus demonstrating that no association with the 5'-boronic function was made. These results suggested that geometric and steric factors have a high influence on the stability of the boronate internucleosidic linkage and also highlighted the importance of natural ribonucleotide partners over modified ones for an effective ligation.

3.4. DNA-Templated Polymerization

Mimicking biological process in the absence of enzymatic activation and controlling the self-assembly of artificial biopolymers is a challenging step in the understanding of the complexity of life but also for the development of biological applications.^[52] In this context, based on the results obtained in DNA-templated ligations, we have designed dynamic systems able to self-assemble through the DNA-templated formation of multiple reversible boronate internucleoside linkage and which could be switched on and off through external stimuli (Figure 10).^[53] For this purpose, bifunctional hexamers containing a boronic acid at the 5'-end and a ribonucleotide at the 3'-end were synthesized and their self-assembly abilities over templates having repeating sections were evaluated by UV thermal denaturation. Control experiments performed in the presence of templates of increasing length on which 1 to 5 unmodified hexamers could be associated revealed that the melting temperature increased slightly as the length of the template increased, probably due to molecular cooperation and stacking interactions. On the other hand, the stability of this assembly decreased with an increase in the pH of the solution (ΔT_m ranging from -1.7°C

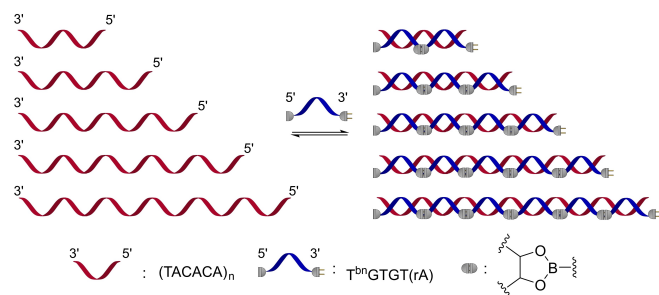


Figure 10. Dynamic DNA-templated polymerization.

to -4.8°C). Under the same conditions, a stabilization of 5.1°C to 11.1°C was observed at pH 7.5 in the presence of bifunctional hexamers. This stabilization was even more marked at pH 9.5 with ΔT_m ranging from $+11.0^\circ\text{C}$ to $+18.8^\circ\text{C}$ for the systems containing one to five boronate linkages respectively. Even higher levels of stabilization were obtained at pH 7.5 with the corresponding RNA templates (ΔT_m ranging from $+15.9^\circ\text{C}$ to $+23.0^\circ\text{C}$) thus confirming the observation made in the DNA-templated ligation experiments.^[50]

In order to identify the structural parameters and the minimal complexity required for the self-assembly of these bifunctional monomers, the system was evaluated using shorter sequences.^[54] This was probed through thermal-denaturation studies at different pH values with DNA templates having three successive repeating section complementary to bifunctional 5-mer, 4-mer or 3-mer. In any case, none of the unmodified sequences resulted in an observable transition at any pH. In the case of bifunctional shortmers a transition at $T_m=9.8^\circ\text{C}$ could be observed with the 5-mer at pH 7.5 that increased to 14.9°C at pH 9.5. By contrast, no transition could be observed with the 4-mer at pH 7.5 but a low T_m value of 7.2°C could be measured at pH 9.5. Finally, with the 3-mer no transition was observed even with an important excess indicating that the equilibrium was largely shifted toward the non-hybridized boronic acid sequence. These results showed that bifunctional trimers represent the current limit for the templated polymerization through the formation of boronate internucleosidic linkage using bifunctional monomers.

The specificity of the assembly was then evaluated with templates having sections complementary to two different bifunctional hexamers. We were pleased to observe that the system was able to transfer the information carried by the templates to the bifunctional hexamers. Indeed, transitions are observed only when the ratio of different hexamers (1:4 or 2:3) matched the numbers of sections featured by the templates (Figure 11). Similarly, the presence of a mismatch at the center of one section of a template prevented the formation of the corresponding boronate internucleoside linkage.^[53,54] Finally, the reversibility of these systems was then investigated by inducing repeating pH variations from 5.5 to 9.5. While no

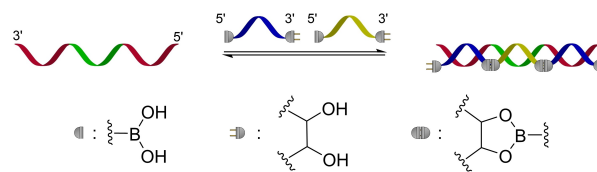


Figure 11. Dynamic DNA-templated polymerization of two different shortmers.

significant change was observed with non-modified hexamers, ΔT_m variations measured in presence of boronic acid-modified sequences were constant after 3 cycles thus demonstrating the ability of the system to be repeatedly assembled and disassembled in the absence of enzymatic activation and in response to pH stimuli.

Based on the formation of reversible and pH-responsive boronate internucleosidic linkage, we have developed an efficient non-enzymatic and sequence specific polymerization process.

4. RNA-Templated Chemical Ligation

Having demonstrated the efficiency of DNA- or RNA-templated assembly of short 5'-boronic acid modified DNA oligomers, we then envisioned to apply the concept to 5'-boronic acid modified RNA sequences. We thus had to synthesize U^{bn} , the analogous RNA residue of T^{bn} . The synthesis of U^{bn} **36** was achieved in 8 steps with 21% overall yield using the 2'-*O*-PivOM strategy developed in our laboratory (Figure 12).^[55,56] The synthesis started by silylation of the 3'-hydroxyl group of commercially available 2'-*O*-pivaloyloxymethyl-5'-*O*-dimethoxytrityluridine followed by detritylation of the 5'-position. The 5'-alcohol was then oxidized by IBX and the resulting aldehyde was converted to alkene **33** through a Tebbe olefination. The borylation step was then realised with *in situ* prepared diisopinocampheylbor-

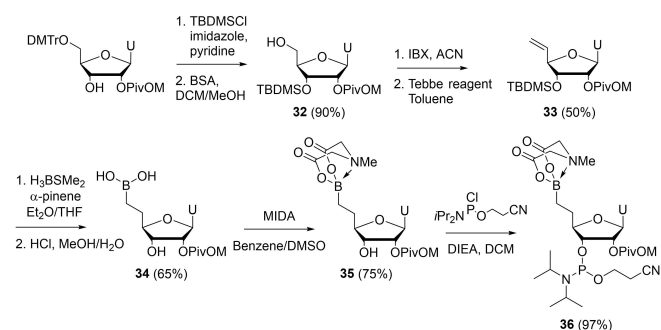


Figure 12. Synthesis of the boronouridine phosphoramidite **36**.

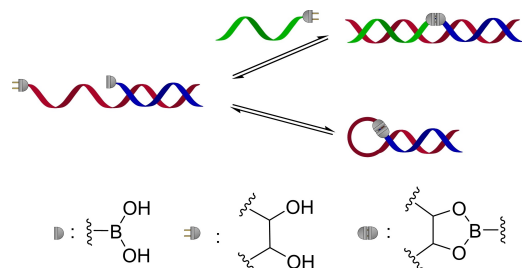


Figure 13. RNA-templated ligation or loop formation.

ane and the 3'-position was deprotected under acidic conditions. Finally, the boronic acid was protected as a MIDA boronate and the 3'-position was phosphitylated to furnish phosphoramidite **36**.

Phosphoramidite **36** was then incorporated at the 5'-end of RNA sequences and we evaluated its ability to generate boronate-like internucleoside bonds with a 7-mer RNA partner in the presence of a DNA or RNA template (Figure 13). As expected, thermal denaturation analysis showed a pH-dependent transition in the presence of the boronic acid-modified sequence and a RNA template.

Surprisingly, a thermal denaturation analysis performed in the presence of the RNA template and the U_6U^{bn} strand but in the absence of the diol partner gave a melting temperature 5.3 °C higher than the unmodified derivative.

We thus hypothesized that the boronouridine was able to interact with the terminal 2',3'-dihydroxy group of the template. This was confirmed by using a template containing a 2'-deoxy or 2'-OMe residue at its 3'-end. In these cases, thermal denaturation analysis did not produce any difference between the modified and unmodified sequences. Varying the 2'-OMe position along the template confirmed that the ligation happened with the *cis*-diol group at the 3'-extremity inducing the formation of a hairpin with 7 unpaired bases (Figure 13). This loop formation was also demonstrated using a DNA template having a ribonucleotide at the 3'-extremity. Molecular dynamics studies confirmed the formation of a hairpin like structure which would be stabilized by hydrogen bonds between nucleotides. This kind of structures are widely found in nucleic acid secondary structure and play key roles in a large number of biological processes.^[57,58] Thus, the reversible formation of boronate internucleosidic linkage could mimic a path from small oligonucleotides to larger and functional biomolecules.

5. Boronate Ester-Based Macrocycles

Encouraged by these results we envisioned the formation of macrocyclic structures by the head-to-tail assembly of a bifunctional precursor containing both a boronic acid and the natural 2',3'-diol of ribose. Boronate-based macrocycles are generally obtained by dehydration of a mixture containing a molecule functionalized by a di-boronic acid moiety and a polyol.^[59,60] In contrast, the formation of macrocycles by head-to-tail assembly of a bifunctional molecule is rather rare.^[61,62] Toward this end, bifunctional boronouridine **38** was synthesized in 4 steps with 23% overall yield (Figure 14).^[63] The synthesis started from commercially available 2',3'-*O*-isopropylideneuridine which was oxidated by IBX and methylenated. Removal of the isopropylidene group was followed by borylation leading to **38**.

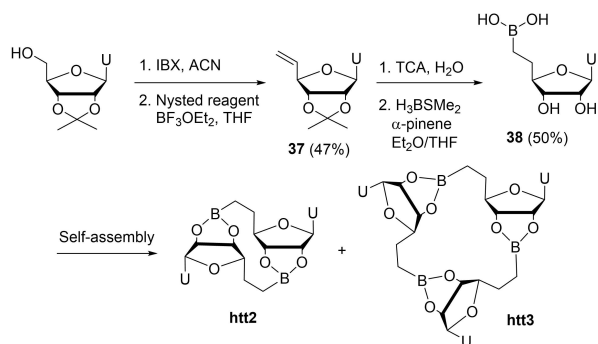


Figure 14. Synthesis of boronouridine and formation of macrocycles.

The self-assembly of compound **38** was first studied by ^1H NMR in D_2O . While at pD 6.4 only the free boronouridine was observed, the increase of the pD value to 9.3 led to a progressive splitting and broadening of the resonances which were attributed to a bound form. Indeed, H_2 , H_3 , and H_6 protons were the most affected indicating a change of hybridization of the boron atom induced by the formation of a boronate ester. The reversibility of the system was confirmed by decreasing the pD to 6.4 leading to the initial spectrum. However, the detailed structure was difficult to determine due to the presence of broad signals. The assembly was thus studied in anhydrous DMF-d_7 , a solvent expected to promote the ligation reaction between the two entities. Two datasets in a ratio 2:1 were identified on the ^1H NMR spectrum and the slow-exchange between the two species was determined by selective saturation experiments. The disappearance of characteristic signals of the monomer **38** on ^1H and ^{13}C NMR spectra indicated the formation of a boronate ester and the reversibility of the system was proved by adding small aliquots of water leading to the initial spectrum. ^{11}B NMR was also investigated and confirmed the formation of a bound structure. Indeed, in anhydrous DMF-d_7 a broad signal was observed corresponding to the trigonal geometry of the boron atom whereas in wet conditions a slight signal corresponding to the boronic acid was obtained. These first results suggested the presence of cyclic assemblies rather than oligomers.

Diffusion ordered spectroscopy (DOSY) experiments were then carried out in order to evaluate the molecular weight of each structure which were estimated to be 467 and 730 g/mol for the major and minor species respectively. These values were in agreement with the formation a dimeric (**htt₂**) and a trimeric (**htt₃**) head-to-tail macrocycles (500 and 750 g/mol respectively, Figure 14). Reference linear and cyclic dimer and trimer oligonucleotides were also synthesized^[64] and the comparison of their diffusion coefficients confirmed the structure of the two head-to-tail macrocycles. The sugar conformation of **htt₂** was then determined by analysis of the vicinal coupling constants and molecular modelling and it

appeared that the sugar was frozen in a rigid North $\text{C}_{4'}$ -*exo* conformation. The formation of a fused five-membered ring bridging the 2' and 3' oxygen atoms as well as the 5'-2',3' head-to-tail cyclization clearly constrained the ribose, thus reducing the pucker amplitude compared to that commonly found in nucleosides and nucleotides.

6. Biological Studies and Applications

6.1. Boronucleotides as Substrates for Human NMP Kinases

The phosphorylation of nucleosides and nucleotides is a central process in biological pathways. Indeed, nucleosides are mono-, di- and triphosphorylated by nucleoside kinases leading to nucleotide triphosphates which are selected by DNA polymerases for DNA synthesis, replication and repair.^[65] These enzymes are thus essential for proper cellular function and represent major targets against infectious diseases caused by DNA and RNA viruses. In this context, modified analogues have acquired an important role as therapeutic agents. We thus decided to study the ability of boronic acid-modified nucleotides to be reversible substrates for human nucleoside monophosphate (NMP) kinase and eventually foil the phosphorylation process.^[66] To this end, boronucleotides **17**, **18**, **19** and **23**,^[43,44] as well as analogues **39**, **40** and **41**, synthesized *via* alkene borylation or cross-metathesis reactions, were used to evaluate the importance of the base, the sugar and the rotational flexibility of the C_5 - C_6 bond (Figure 15).

All compounds were screened as substrates towards the five human NMP kinases which are known for their high specificity of action. It appeared that boronothymidine **17** was the sole analogue to act as a substrate of human TMP kinase and thus to be phosphorylated through the formation of B–O–P bonds. These results demonstrated the importance of a close mimicry of the natural NMP for optimal recognition within the active site of the kinase and consequently phosphorylation. Concerning the other derivatives, none of

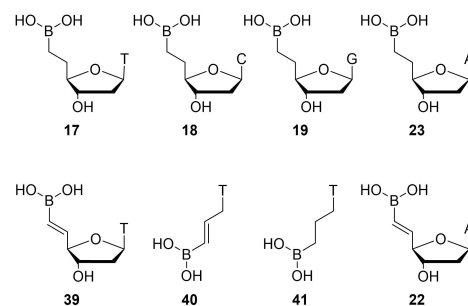


Figure 15. Structures of the boronucleotides used as substrates for human NMP kinases.

them was able to bind to their corresponding kinases which indicated that each kinase recognizes its substrate *via* different criteria. However, all compounds showed moderate to good levels of inhibitory activity which suggested that these analogues could be accommodated in the active site of targeted NMP kinases. Unfortunately, due to their weak affinity the reaction mechanism was not studied.

Conformational changes induced by these substrates were then investigated by circular dichroism and it appeared that all analogues were able to bind human NMP kinases with different conformational changes depending of the intrinsic enzyme conformation. Indeed, unsaturated compounds **39** and **40** led to important conformational changes compared to saturated analogues **17** and **41**. These results underlined the different binding modes of the derivatives with the human NMP kinases. Interestingly, the properties of nucleotide-binding sites of the enzymes were studied by fluorescence with mant-ATP (Methylantraniloyl-ATP) leading to a blue shift after displacement of the fluorophore by the substrates and it appeared that some of them were able to bind ATP site which was not the targeted site. Docking experiments were also performed in order to illustrate the binding mode with boronothymidine **17** and boronocytidine **18** as examples. In the first case, the base moieties of **17** and its natural counterpart were quite well superimposed while sugar-phosphate moieties differ because of the partial hydrophobic character and flexibility of the boronic acid group. However, the boronocytidine **18** adopted a completely opposite position preventing any phosphorylation.

These results thus demonstrated that borononucleotides could accommodate the NMP site of selected human NMP kinases. Unfortunately, no antiviral activity was found on cancer cells or DNA viruses but it still suggests potential development of new monophosphate mimics as potential substrates for NMP kinases.

6.2. Stability of Boronic Acid Modified Oligonucleotides Towards Nucleases

To fully exploit the potential of boron-modified oligonucleotides, we also investigated the resistance of 5'-boronic acid modified sequences toward nucleases degradation both at the single and double stranded levels.^[67,68] Among the many nucleases that were evaluated, snake venom phosphodiesterase (SVPD) and calf spleen phosphodiesterase (CSPD) provided the most interesting results. Three systems were evaluated: a single strand 12-mer modified by a boronic acid at its 5'-end, this same single strand hybridized to a 21-mer template and finally a duplex presenting a boronate junction realized between the 12-mer 5'-boronic and a 3'-ribonucleotide sequence both hybridized to the 21-mer template. As expected all unmodified control sequences were degraded in less than

five minutes whether in single or double stranded strand. Interestingly, 5'-boronic acid modified single-stranded sequences showed some resistances toward SPVD ($t_{1/2}$ =180 min). The stability was then studied in duplexes in the presence of a DNA template. Again, the non-modified duplex was rapidly degraded whereas the duplex with the boronic acid-containing sequence showed good stability ($t_{1/2}$ =180 min for the modified 12-mer and 75 min for the template). The influence of a boronate internucleosidic linkage was then evaluated by mixing the template, the 5'-boronic acid modified sequence and the 3'-ribonucleotide complementary sequence. While the unmodified duplex was digested rapidly, the presence of a boronate internucleosidic linkage led to a good resistance to enzymatic degradation ($t_{1/2}$ =75 min for the template and 180 min for both complementary strands). This observation suggests that the boronate junction remains intact in the absence of the template. Those results were even more marked with CSPD. Indeed, the stability was increased whether with the boronic acid modified single strand ($t_{1/2}$ >5 d) or with the duplex ($t_{1/2}$ >5 d for the modified sequence and 24 h for the template). Since CSPD is inhibited by 5'-phosphate groups it was not totally unexpected that as phosphates isosteres the 5'-boronic acid-modified sequences were not degraded. However, the enhanced stability of the unmodified template suggested a protective effect of the boronic acid function toward degradation. Finally, with the duplex composed of the template, the 5'-boronic acid modified sequence and the 3'-ribonucleotide sequence a high resistance was observed ($t_{1/2}$ >5 d for the two complementary sequences and 24 h for the template). Once again, the boronate junction induced a protective effect on the template and confirmed the existence of such junction in the absence of a template.

The ability of the boronic acid function to protect the template from digestion led us to envision the development of a label-free fluorescence-based strategy for the detection of single-point mutations in DNA using the SYBR Green I, an intercalator exhibiting high fluorescence enhancement upon binding with a DNA duplex (Figure 16). We selected the well-characterized A→G mutation of the Cystic Fibrosis Transmembrane conductance Regulatory (CFTR) gene as a model and demonstrated that when 5'-boronic acid was placed opposite to point mutation at the N+4 position and in the presence of a 3'-ribonucleotide partner the presence of a mismatch induced a significant increase in the degradation of the target sequence by SVPDE. The degradation was monitored by fluorescence and a 17-fold difference of intensity was recorded between the wild-type and mutant thus demonstrating the ability of boronic acid probes to discriminate sequence differing by only one residue (Figure 16).

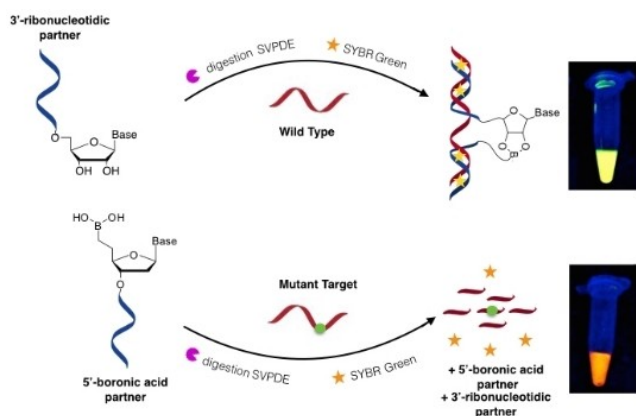


Figure 16. Label-free detection of single point mutations

6.3. Stability of Boronic Acid Modified Oligonucleotides Towards RNase H

Among the different phosphodiesterases evaluated in our biocompatibility studies we were also interested in ribonuclease H (RNase H) which is an intracellular enzyme able to specifically degrade a RNA strand of a RNA/DNA hybrid duplex. Native PAGE analysis showed that 5'-boronothymidine modified oligonucleotides were able to inhibit RNase H activity.^[69] Considering the ability of oxidant species to oxidize boronic acids into the corresponding alcohol we designed a stimuli-responsive system able to detect peroxynitrite, a power-

ful oxidant produced in cells. For that purpose we introduced a quencher at the 3'-end of a boronic acid-modified oligonucleotide and a fluorophore at the 5'-end of a complementary 14-mer. We showed that upon treatment with increasing concentration of peroxynitrite, an increase of fluorescence is observed with a limit of detection of 28 nM. Satisfyingly, when implemented in RAW267.4 murine macrophages known to produce ONOO^- via immunogenic stimuli and transfected with our modified duplex, a significant and specific fluorescence enhancement was observed (Figure 17a). Considering the critical role of RNase H in cellular processes this system could allow the development of sensors for the detection of reactive oxygen species in living cells. While 5'-boronic acid modified oligonucleotides hybridized to a RNA target inhibits RNase H activity we further demonstrated that the addition of a 3'-ended diol partner was able to reactivate the endonuclease activity. This specificity was exploited by the development of an on/off-switched system thanks to the formation of a reversible boronate internucleosidic linkage (Figure 17b).^[70] Molecular dynamics studies demonstrated that the boronate ester function achieved a critical interaction with the side chain of the amino acids within RNase H phosphate binding pocket. The activation of the RNase H was then studied with various diol partners and it appeared that the spatial confinement and specific interactions with RNA also influenced the binding affinity. While RNase H does not exhibit sequence-specificity, the use of a boronate internucleosidic linkage allowed the development of an innovative

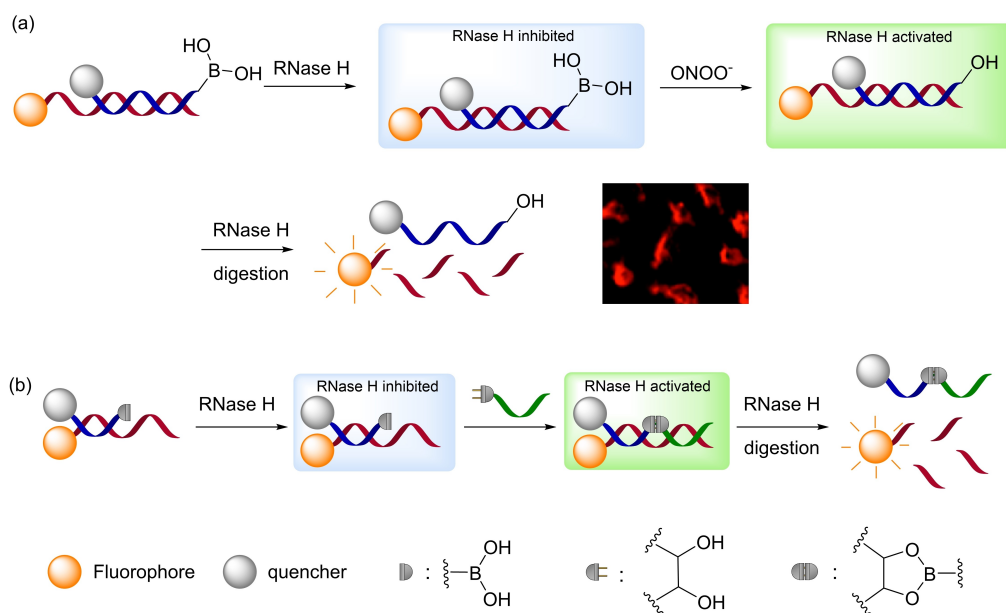


Figure 17. (a) RNase H-mediated imaging of peroxynitrite *via* 5'-boronic acid modified oligonucleotides. (b) RNase H activation *via* the formation of a boronate internucleosidic linkage.

approach for controlling RNase H activity that was used for mismatch discrimination.

6.4. Boronic Acid-Mediated Control of Split-DNAzyme Activity

Inspired by all these results, we decided to evaluate the ability of engineered boronate internucleosidic linkage to control DNAzymes activities.^[71] The most prominent representatives of the DNAzyme family are RNA-cleaving DNAzymes which are composed of a catalytic core flanking by two substrate-recognition domains that catalyze the phosphodiester bond cleavage of their RNA substrate in presence of a divalent cation.^[72,73] Recently, split DNAzymes that consist in dividing the parent DNAzyme in two or more non-functional fragments able to bind in presence of a specific target have emerged as a new generation of biosensors with improved stabilities and catalytic activities.^[74] Although these short systems are easier to synthesize and carry fewer negative charges per strand, it can be challenging to efficiently design stable and active systems. This drawback can be counterbalanced by the use of modified fragments that upon addition of the target are brought into close proximity to allow covalent end joining. Among the existing DNAzymes, we selected as a model the 10–23 DNAzyme which is a catalytic oligonucleotide able to cleave a RNA substrate in a sequence-specific fashion in the presence of Mg^{2+} ions. New split 10–23 DNAzymes were designed by splitting the parent DNAzyme into two fragments individually at all thymidine positions, located either in the catalytic loop or in one of the flanking arms, and the RNA substrate was fluorescently labelled with ATTO₆₈₀ at its 3'-end for denaturing polyacrylamide gel electrophoresis (PAGE) analysis. The first fragment of each system carried a ribonucleotide at its 3'-end while the second one was modified by a boronic acid at its 5'-end (Figure 18). The 5'-boronothymidine **17** was incorporated at each thymidine positions to evaluate the impact of the flexibility and of the π - π stacking interactions.

A systematic evaluation with split systems allowed to identify the best-performing system to restore the DNAzyme activity. While the splitting led to a strong decrease or even an inhibition of the catalytic activity of unmodified systems, the spontaneous assembly of the two modified fragments restored the activity of the stabilized DNAzyme. By varying the position of the boronate ester we were able to characterize the optimum split site for maintaining high levels of RNA cleavage. In the long term, the reversible formation of boronate internucleoside linkage may be a promising approach to control functional nucleic acid assembly for the development of valuable tools for biosensing.

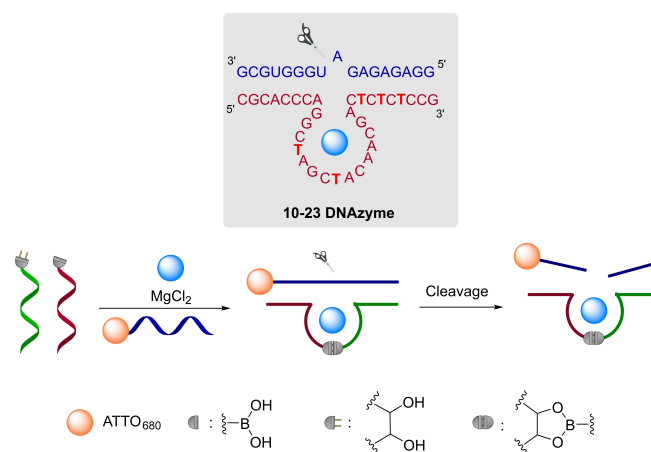


Figure 18. (a) Boronic acid-mediated assembly of functional split 10–23 DNAzymes. Thymidine residues that have been replaced are highlighted in red.

7. Selective Chemical Modification of DNA and RNA with Boronic Acids

Nucleic acids are part of the three major biopolymers that are essential and common for all known forms of life and thus constitute a very broad domain with numerous therapeutic or diagnostic applications.^[75] However, it could be necessary to modify them in order to respond to structural and functional properties as stability, reversibility or assembly efficacy. More recently, modified oligonucleotides were also widely used for drug discovery through DNA-encoded libraries.^[76] In this context, the development of DNA-compatible chemistry is necessary. Modified oligonucleotides are classically synthesized by automated synthesis with appropriate phosphoramidite building blocks or by enzymatic chain assembly with nucleoside triphosphates. For both approaches, a multistep synthesis of the modified monomer is required which has to tolerate reagents and conditions used for the elongation. Interestingly, the introduction of boronic acid groups into oligonucleotides remains challenging especially because of the particular reactivity of this function. Enzymatic polymerization,^[77,78] amide bond formation^[79] or strain-promoted azide-alkyne cycloaddition (SPAAC)^[80,81] were already developed but all of them required the multistep synthesis of modified nucleosides. We thus developed two rapid, efficient and modular methods for post-synthetic introduction of a boronic acid at the 5'-extremity (Figure 19a) or into the DNA sequence (Figure 19b) through on-column copper-catalysed azide-alkyne cycloaddition (CuAAC) reaction.^[82] The first method implied the reaction between a 5'-azido oligonucleotide obtained in two steps from the corresponding alcohol and 4-ethynylphenylboronic acid pinacol ester. For the second method, a 2'-*O*-propargyluridine was incorporated at the desired position into

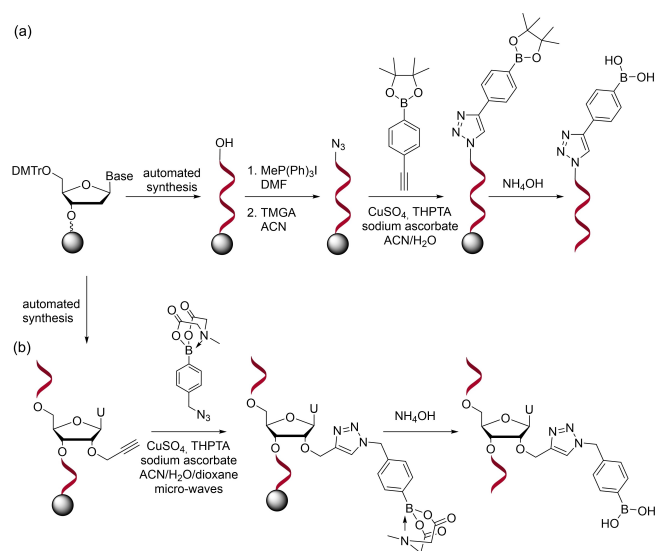


Figure 19. On-column CuAAC conjugation of boronic acids (a) at the 5'-end or (b) inside DNA and RNA sequences. (TMGA : tetramethylguanidinium azide; THPTA : Tris(3-hydroxypropyltriazolylmethyl)amine).

the DNA sequence and the CuAAC reaction was realized in presence of 4-(azidomethyl)phenylboronic acid MIDA ester. While the insertion of copper(I) between carbon-boron bonds is a well-documented side reaction that can hamper the efficiency of the CuAAC reaction,^[79,81,83,84] our group already reported that adequately protected boronic acid could withstand the CuAAC reaction conditions.^[85] The two on-column CuAAC reactions were applied on DNA and RNA sequences (up to 20 residues) leading to boronic acid-modified oligonucleotides with good yields. The presence of the boronic acid moiety was confirmed by application of the Alizarin Red S (ARS) displacement assay.^[86,87] The development of these two new methods increased significantly the repertoire of possible DNA and RNA conjugation chemistries and could find use for the design of functional oligonucleotides, notably for the development of saccharide or *cis*-diols-containing molecules sensors.

8. Conclusion

Our work began with the desire to bring together the characteristics of boronic acids and the specificities of nucleic acids in a single research project. Based on the ability of boronic acids to bind with *cis*-diol partners, we started our journey by developing fluorescence methods for the detection of nucleosides of interest that do not carry chromophore groups. We then started a synthetic work that led to the synthesis of a series of 5'-boronic acid nucleotides mimicking nucleotides monophosphate. These analogues, which we named boronucleotides, allowed the controlled and rever-

sible formation of a new boronate internucleoside linkage for the development of functional oligonucleotides that could find various applications in biotechnologies. The desire to mimic biological processes in the absence of any activation and to generate dynamic systems has led us to develop innovative processes mimicking ligation and polymerization in the absence of enzymes. This was not done without difficulty, far from it! Indeed, the reversible nature of the boronic acid/boronate equilibrium does not allow their detection by methods commonly used in nucleic acid chemistry. It is for example extremely difficult to characterize these assemblies by PAGE, even in non-denaturing conditions because the resulting ligated duplexes are not stable enough to be observed, especially the short ones. This is also true under standard HPLC conditions. Similarly, MALDI-TOF analysis generally used to characterize oligonucleotides do not represent a reliable analytical method to probe the formation of boronate junction because the latter can be generated within the source. However, thermal denaturation studies represent a method of choice to validate the formation of boronate linkages. The pH-dependent stabilization observed, associated with multiple control experiments with sequences lacking the 3'-end diol unit allowed us to demonstrate unambiguously the reversible formation of boronate linkages.

The capacity of 5'-end boronic acid-modified oligonucleotides to induce high resistances to degradation by nucleases led to the development of an innovative fluorescence-based method for single mismatch detection as well as peroxy nitrite imaging in living cells. More recently, boronate ester formation has been used to control the assembly and the activity of functional architectures. The results obtained with split-DNAzymes allows us to consider the design of more complex structures displaying a variety of functions.

The immediate outlook of this research implies the synthesis of the four boronoribonucleotides and their incorporation into RNA sequences as well as the study of their biocompatibility with various enzymes. Indeed, the ability of boronoribonucleotides to self-assemble and generate macrocycles in the absence of chemical or enzymatical activation set the stage of investigating the formation of macrocycles from a mixture of pyrimidines and purine 5'-boronic acid analogues. Beyond the formation of macrocycles, this study underlines the capacity of these nucleosides analogues to self-assemble and allows to envision for further future outlook the dynamic templated assembly of boronoribonucleotide residues to engineer oligonucleotides fully modified with boronate linkages. Evolving artificial reversible backbone linkages able to respond to external stimuli will thus approach more fundamental questions like the origin and evolution of life.

Overall, this work has demonstrated the potential of coupling boronic acid to nucleic acids for applications in

detection, bioconjugation or for the development of bioinspired functional assemblies.

We will continue our efforts with more in-depth implementations and exciting new developments in this area.

Acknowledgements

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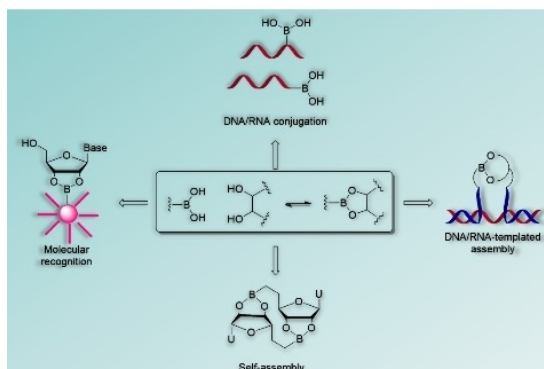
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PERSONAL ACCOUNT



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Applications of the Reversible Boronic Acids/Boronate Switch to Nucleic Acids

This personal account summarizes the research carried out in our laboratory at

the crossroads of nucleic acids and boronic acids chemistries.
