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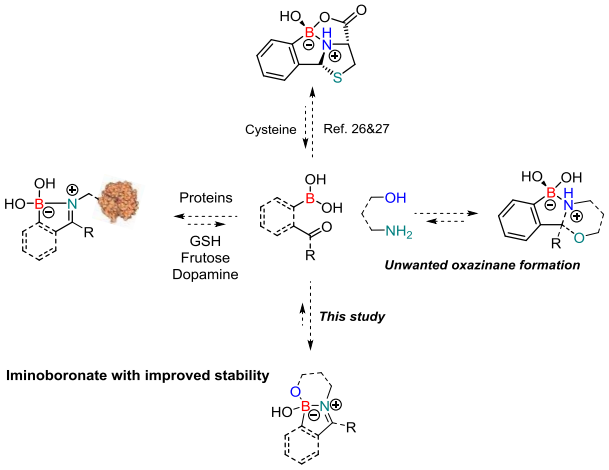
N,O-Iminoboronates: Reversible Iminoboronates with Improved Stability for Cancer Cells Targeted Delivery

Ricardo M. R. M. Lopes,[a] Ana E. Ventura,[a,b] Liana C. Silva,[a,b] Hélio Faustino,*[a] Pedro M. P. Gois,*[a]

Abstract: Herein we present a new class of iminoboronates obtained from 2-acetylbenzene boronic acids and aminophenols. The *N,O*-ligand topology enabled the formation of an additional B-O bond that locks the boron centre in a tetrahedral geometry. This molecular arrangement decisively contributes to improve the construct stability in biocompatible conditions, retaining the iminoboronate reversibility in more acidic environments. 2-Acetylbenzene boronic acid was reacted with a fluorescent amino-coumarin to yield a stable and non-fluorescent *N,O*-iminoboronate. This mechanism was further used to assemble a folate receptor targeting conjugate that selectively delivered the fluorescent amino-coumarin to MDA-MB-231 human breast cancer cells.

Nature explores an array of ligation strategies to construct functional molecules that support most of the biological processes essential to life. Noncovalent interactions including hydrogen bonding, electrostatic and hydrophobic interactions, have been long established as the basis for the assembly of reversible molecular complexes that are able to engage in dynamic processes.[1] On the other side of the spectrum, the use of covalent bonds typically generates more stable compounds that often exert their activity based on their structure integrity.[2,3] Merging the stability and reversibility attributes in the same bonding motif is a rarer event. Though disulfide bonds stand as a prominent example in Nature of a covalent bond that under redox regulation is reversible, and because of that, controls important processes such as protein folding.[4] Recent appreciation for the unique properties of reversible-covalent bonds resulted in their use to design enzyme inhibitors, dynamic covalent libraries or stimulus responsive multivalent molecules.[5] Iminoboronates are a class of reversible-covalent based compounds in which an imine is stabilized by the coordination with an adjacent boronic acid.[6–13] In 2012 we used this strategy to perform the reversible functionalization of the ε-amino group of lysine residues exposed on the surface of proteins,[14] and later, to generate cancer cell targeting folic-acid fluorescent conjugates.[15] Since then, we and others have used iminoboronates in areas as diverse as bioconjugation,[16,17] synthesis of responsive materials[18–20] or in the discovery of innovative inhibitors of protein-protein interactions.[21] One of the most important attributes of iminoboronates is their inherent reversibility, which can be accelerated by endogenous molecules like glutathione (GSH), fructose or dopamine.[6,8,14,22] However, recent studies showed that iminoboronates also hydrolyse spontaneously under physiological conditions.[23,24] This lack of stability constitutes an important limitation, when envisioning the use of this reversible linkage in the construction of stimulus responsive conjugates, to deliver cargo selectively to cancer cells.[8,25] Based on this issue, we set-out to improve the stability of iminoboronates aiming at their use in the synthesis of reversible linkers for targeting conjugates.

Recently we and Gao independently observed that the reaction of 2-formylbenzene boronic acid (2FBBA) with cysteine generates a fused tricyclic structure due to the formation of an additional reversible bond between the boron atom and the carboxyl group.[26,27] Density functional theory calculations performed on this system have revealed that this B-O bond contributes decisively to the stabilization of the product but does not compromise the system's reversibility.[27] In this context, Bane also described the formation of stabilized hydrazones using α-amine carbohydrazides and 2-formylbenzene boronic acids.[28] Based on these observations, we envisioned that a strategy to improve these construct's stability would be to design a *N,O*-bidentate ligand that enables the formation of the iminoboronate with an additional B-O bond Scheme 1.



Scheme 1. Left: Previously reported iminoboronate formation. Top: Thiazolidine formation with additional bond between the boron and the carboxylic acid. Right: unwanted oxazinane formation. Bottom: current proposal for iminoboronate formation with improved stability.

To test this idea we selected as prerequisites for the ligand design the presence of a primary aliphatic amine to form the iminoboronate and a hydroxyl function with improved reactivity towards the boron centre (Scheme 1). Based on these

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requirements, we selected aminophenols **1** and **2** as prime candidates to generate *N,O*-iminoboronates because they exhibit the required nucleophiles and are able to accommodate the N-B-O bonds in a more rigid six member ring. Therefore, the synthesis of aminophenols **1** and **2** (figure 1) was performed following reported methodologies.[29,30]

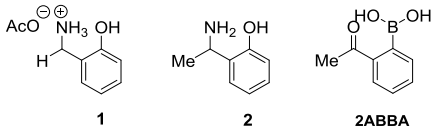
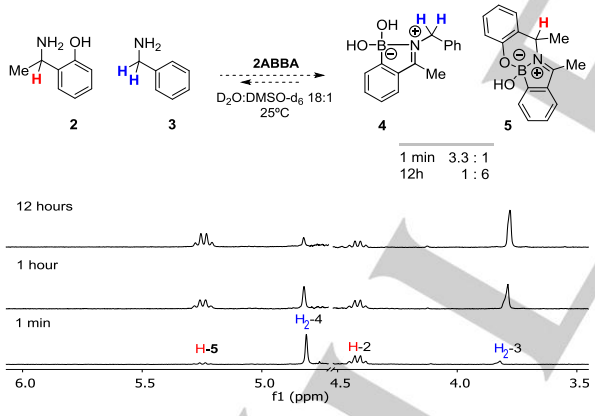


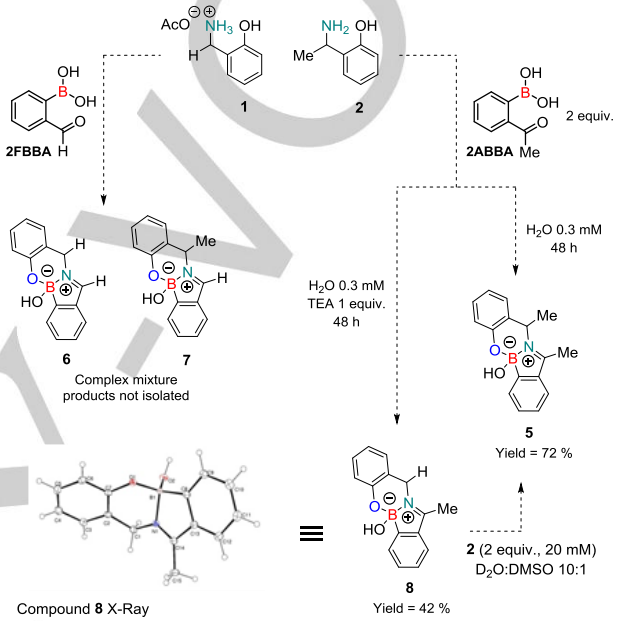
Figure 1. Selected aminophenols and 2ABBA.

Once prepared, we tested if this amine topology could render the desired *N,O*-iminoboronates. To study this, a competition experiment was performed in which equimolar amounts of benzylamine **3** and aminophenol **2** were added to a solution of 2-acetylbenzene boronic acid (2ABBA) in D_2O:DMSO-d_6. The reaction was monitored by ^{1}H-NMR and the analysis of the spectra depicted in Scheme 2, revealed that just after one 1 min. the 2ABBA component was completly consumed to produce a 3.3:1 mixture of iminoboronates **4** and **5** respectivly. Gradually, this ratio shifted towards the formation of **5**, and after 12h, the *N,O*-iminoboronate become the major product (ratio 1:6 of **4** to **5**) in the mixture. These results clearly indicate that, although the iminoboronate **4** is kineticaly favoured, the improved stability promoted by the *N,O*-bidentate ligand shifts the equilibrium to the formation of the more stable *N,O*-iminoboronate **5**. Under these conditions, the formation of oxazinane was never observed.



Scheme 2.Competition experiment between **2** (1.5 equiv.) and **3** (1.5 equiv.) in the presence of 2ABBA (22 mM). The solvent residual peak of HOD was removed for clarity; similar results were obtained in deuterated PBS pH 7.4 (see supporting information for the full ^{1}H-NMR spectra).

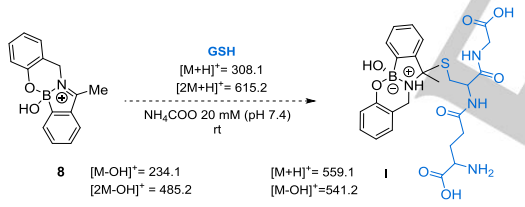
Next, different *N,O*-iminoboronates were synthesized aiming at the evaluation of their stability and reversibility properties. This study was initiated with the reaction of aminophenols **1** and **2** with 2FBBA in aqueous media. Unfortunately, although it was possible to detected by ^{1}H-NMR the presence of the expected *N,O*-iminoboronates **6** and **7** in the reaction crude mixture, this reaction generated an inseparable mixture of products.[31] Hence, the same transformation was attempted with 2ABBA. Gratifyingly, as shown in Scheme 3, the reaction with aminophenol **1** afforded **8** in 42% yield under basic conditions while the condensation with **2**, proceeded smoothly in water to afford the *N,O*-iminoboronate **5** in 72% yield. Suitable crystals for X-ray analysis were obtained for *N,O*-iminoboronate **8**. As shown in Scheme 3, the structure of **8** confirmed the expected fused bicyclic unit comprising the iminoboronate and the formation of the B-O bond that locks the boron centre in a tetrahedral geometry.



Scheme 3. Reactions of 2ABBA or 2FBBA (2 equiv.) with aminophenols **1** and **2** in water (0.3 mM). ORTEP-3 diagram of **8**, using 30% probability level ellipsoids. CCDC 1823602 (**8**) contain the supplementary crystallographic data for this paper. These data are provided free of charge by the Cambridge Crystallographic Data Centre.

Once established the preparation of *N,O*-Iminoboronates, their stability was evaluated in different biocompatible medias. In a D_2O:DMSO (5:1) mixture, compound **8** (20mM) proved to be quite stable as no hydrolysis was detected over one week period and upon a 10 fold dilution (2mM) (SI, figures S 3-7). In PBS buffer (0.1 mM) at pH 7.4, *N,O*-Iminoboronate **8** presented less then 10% hydrolysis after 48h, and simillar results were obtained in the presence of frutose and dopamine. Importantly, in human plasma, compound **8** hydrolized less than 20% after 48h (SI, tables S2 - S8 and figures S20 - S26). Encouraged by these results, we next studied if these constructs could still engage in reversible mechanisms. Therefore, compound **8** (20mM) was submitted to a cross reactivity experiment with aminophenol **2** (2 equiv., D_2O:DMSO). Gratifyingly, despite been a slow process, under these conditions **8** slowly converted into **5** (50% conversion in 5 days). These results indicate that the additiononal B-O bond contributed to improve the iminoboronate stability but, very importantly, did not preclude its reversible nature.

Motivated by these results, we questioned if *N,O*-Iminoboronates would exhibit a biocompatible mechanism of reversibility that could be explored for targeted delivery. Cancer cells are well known to exhibit moderately acidic subcellular compartments (early endosomes – pH 6.3 and lysosomes – pH 4.5) and higher concentrations of cytoplasmic glutathione (GSH).[18] These particular characteristics are often explored to promote the intracelluar dissociation of functional conjugates. Therefore, the stability of compound **8** was studyed in PBS (1mM) at different pHs and in the presence of GSH. Regarding the pH, compound **8** exhibit less than 20% hydrolysis after 1 week in PBS (1mM) at 6.3 while at lysosomal pH (PBS, pH 4.5) the construct hydrolysed much faster (5.8h half-life), confirming the reversibility of *N,O*-Iminoboronates in more acidic environments. Regarding the stability under reducing conditions, we have recently observed that GSH can promote the hydrolysis of iminoboronate complexes *via* thiol addition to the more electrophilic imine carbon centre.[18] Based on these results, we anticipated that *N,O*-iminoboronates could undergo a similar mechanism of hydrolysis in the presence of GSH. To explore this mechanism, compound **8** (1 mM) was incubated with 10 and 100 equiv. of GSH in ammonium acetate solution (20 mM, pHs 7.4). The analysis of the reaction mixtures by ESI-MS revealed the formation of an adduct of GSH with **8** (tentatively assigned as adduct **I**, $[M+H]^+$= 559; $[M\text{-}OH]^+$=541; Scheme 4 and Figure S9 in SI). Interestingly, despite the formation of adduct **I**, the presence of GSH had no impact on the construct **8** stability in PBS (pH 7.4) probably due to the reversible nature of **I**. Therefore, it is not possible to ascertain the impact of intracellular GSH on the construct hydrolysis.

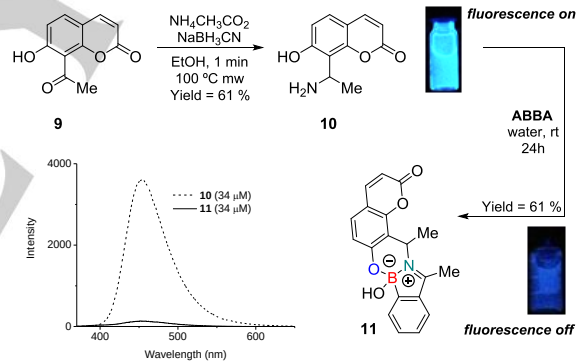


Scheme 4. Conjugate **8** (0.1 mM) in the presence of GSH (1.0 mM).

All together, these results suggest that the intramolecular formation of a B-O bond stabilizes the iminoboronate under aqueous conditions, but it does not preclude the reversible nature of these constructs. Therefore, *N,O*-iminoboronates were next applied in the synthesis of a reversible conjugate to engage in targeted deliver to cancer cells. For the construction of this conjugate, folic acid was selected as the targeting unit because many cancer cells over-express internalizing surface receptors for this small vitamin.[32,33] Regarding the conjugate's cargo, a fluorescent phenolic dye was selected, because we anticipated that the formation of the B-O bond would lead to the probe switch-off, while the construct hydrolysis would restore the probe fluorescence. If successful, this fluorescence *off-on* mechanism would be instrumental to signal the *N,O*-iminoboronate intracellular reversibility. Hence, to test this concept, the amino coumarin **10** was synthesised from 8-acetyl-7-hydroxy-coumarin **9** and then reacted with 2ABBA to yield the expected *N,O*-iminoboronate **11** in 61%. Monitoring the process by UV-Vis spectroscopy under pseudo first order conditions, reveal that this reaction proceeds with a k_2 = 32 ± 4 $M^{-1}h^{-1}$. Then, the photophysical properties of **10** and **11** were evaluated. As shown in Scheme 5, the amino coumarin **10** proved to be highly fluorescent in aqueous media (Φ_f = 0.80) while the *N,O*-iminoboronate formation resulted in a remarkable decrease (Φ_f = 0.025) of the coumarin **10** fluorescence which confirmed the proposed *switch-off* mechanism induced by the formation of the B-O bond.

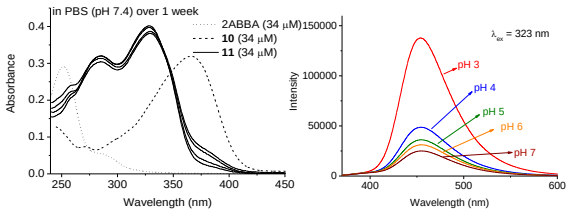
Next the stability and reversibility of **11** was monitored in PBS. As in the previous cases, also the *N,O*-iminoboronate **11** proved to be quite stable under these conditions with less than 12% hydrolysis over 7 days.[34] Differently, when incubated in PBS at lower pHs (3-6), **11** underwent hydrolysis with the concomitant release of the coumarin **10**. This process was confirmed by a notorious enhancement of the fluorescence in the reaction mixture as shown in Scheme 6.

Once established the stability and controlled reversibility of **11**, this bonding motif was used in the assemblage of a cancer cell folate receptor targeting conjugate, featuring an acidic mediated *turn-on* fluorescence. With this objective the 2ABBA component was modified to include an azide group, envisioning the construct post-functionalization *via* a strain promoted azide-alkyne cycloaddition. Next the **14**[16] was reacted with coumarin **10** under aqueous conditions to prepare the *N,O*-iminoboronate **15** in 84% yield. Finally the cyclooctyne-folate targeting unit **16** was prepared based on reported methodologies[35] and installed on **15** to generate the conjugate **17**.

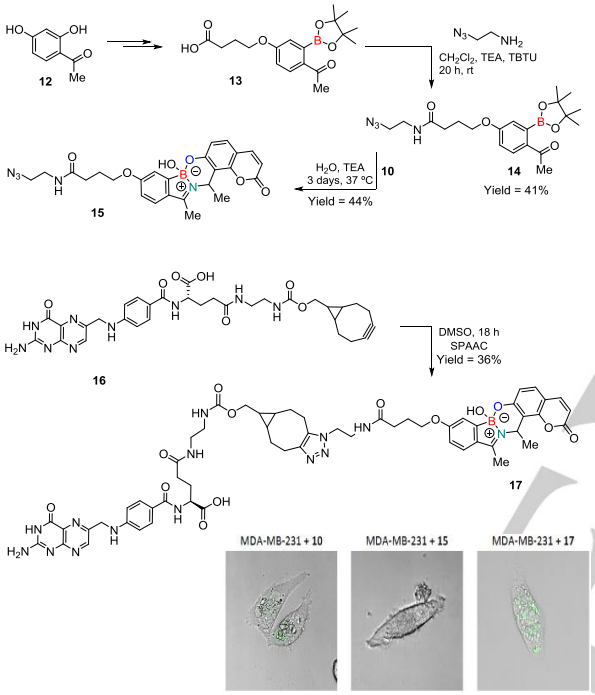


Scheme 5. Top: Synthesis of **11** and fluorescence of **10** (1 mM in water) and **11** (1 mM in water) when irradiated by a 365 nm lamp. Bottom: Fluorescence spectra (λ_{ex} = 323 nm) of **10** (34μM) and **11** (34μM) in PBS pH 7.4.

Finally, the selective delivery of the fluorescent probe to cancer cells was studied using MDA-MB-231 human breast cancer cells that are well known to overexpress folate receptors. Hence, this cell line was treated with conjugate **17** and the internalization progress was monitored by confocal microscopy. Images obtained at 10 and 30 minutes (see SI for details) clearly showed the fluorescent coumarin probe dispersed throughout the cell cytoplasm, confirming the successful iminoboronate entry into the cells and the construct hydrolysis. Differently, the iminoboronate **15** in the same conditions, failed to deliver the fluorescent coumarin. This observation is a good indication of the vectorization induced by the folic acid unit used in conjugate **17**.



Scheme 6. Top: UV − Vis spectra of 2ABBA, **10** and **11** in PBS pH 7.4. Stability of **11** (34 μM) in PBS (pH 7.4) over 1 week. Bottom: Fluorescence spectra (λ_{ex} = 323 nm) of 11 (34 μM) after 1h incubation in PBS at 37 ºC at pH values between 3 and 7.



Scheme 7. Syntheses of the conjugates. Bottom: Confocal fluorescence microscopy analysis of MDA-MB-231 human breast cancer cells: left: incubated with **10**; middle: incubated with **15**; right: incubated with complex **17**.

In summary, here we show that 2-acetylbenzene boronic acids react with 2-(1-aminoethyl)-phenols to generate *N,O*-iminoboronates. This class of iminoboronates exhibits an additional B-O bond that locks the boron centre in a tetrahedral geometry. Differently from iminoboronates prepared with primary amines, *N,O*-iminoboronates are considerable more stable in biocompatible conditions and still reversible in more acidic environments (5.8h half-life in PBS pH 4.5). The *N,O*-iminoboronates' molecular arrangement enabled a new mechanism for controlling the *on-off* fluorescence of an aminocoumarin fluorescent probe *via* the formation and hydrolysis of the B-O bond. The suitable properties of *N,O*-iminoboronates, were applied in the construction of a folate targeting conjugate, featuring an pH mediated *turn-on* fluorescence which was able to selectively deliver the amino coumarin to MDA-MB-231 human breast cancer cells.

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Keywords: boronic acids · drug delivery · small-molecule–drug conjugates · iminoboronate · reversibility

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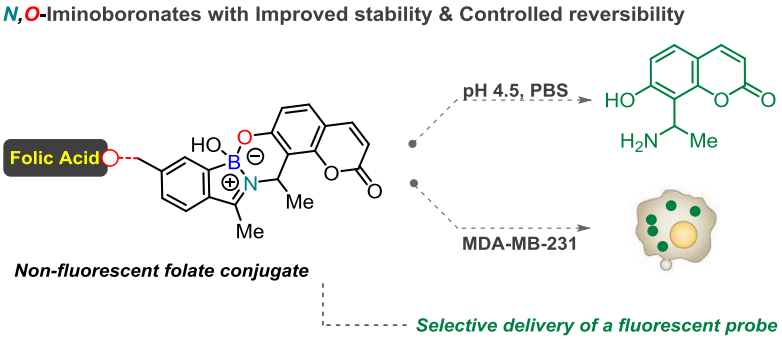

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