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Iminoboronates: A New Strategy for Reversible Protein Modification

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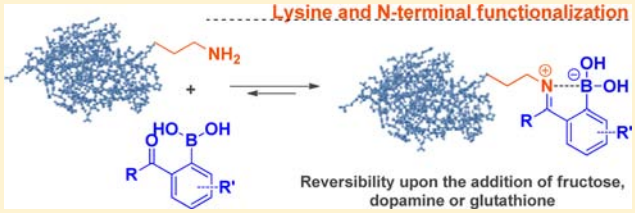
Supporting Information

ABSTRACT: Protein modification has entered the limelight of chemical and biological sciences, since, by appending small molecules into proteins surfaces, fundamental biological and biophysical processes may be studied and even modulated in a physiological context. Herein we present a new strategy to modify the lysine's ε-amino group and the protein's *N*-terminal, based on the formation of stable iminoboronates in aqueous media. This functionality enables the stable and complete modification of these amine groups, which can be reversible upon the addition of fructose, dopamine, or glutathione. A detailed DFT study is also presented to rationalize the observed stability toward hydrolysis of the iminoboronate constructs.

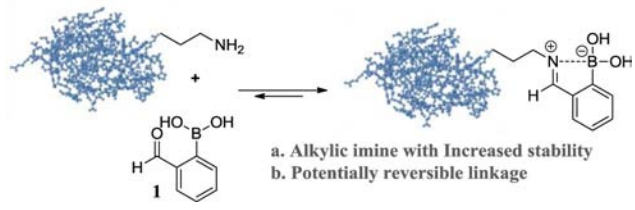


INTRODUCTION

Proteins are unarguably responsible for the majority of functional attributes of living organisms, and many of these functions rely on post-translational modifications at specific sites of proteins, both as part of their function and in response to environmental challenges.[1,2] Therefore, appending small molecules into proteins emerged as a strategy of paramount importance to study fundamental biological processes.[3−6] From the organic chemistry perspective, the marginal stability of proteins *in vitro* is an overwhelming challenge in designing such chemical reactions, as they need to be fast and very selectively conducted with high yields in aqueous media, at low reagent concentration, and at physiological pH, temperature, and pressure.[1−7]

The lysine side chain is involved in many post-translational modifications with essential roles in cell physiology and pathology: methylation, acetylation, biotinylation, glycation, ubiquitination, and sumoylation.[8,9] It is, therefore, with no surprise that lysine residues became such popular targets for modern bioconjugation methodologies.[3,4] The lysine ε-amino group can react with electrophiles such as activated esters, sulfonyl chlorides, isocyanates, or isothiocyanates.[10] Alternatively, the lysine residue can be selectively modified on the basis of the generation of imines, though a second reductive step is commonly necessary to achieve an efficient conjugation, due to the inherent reversibility of this functionality.[11−13] Therefore, the formation of stable imines in aqueous media would allow a direct, selective, and potentially reversible strategy to modify the lysine residues and *N*-terminal.[14−17] In this context, we envisioned that such imines could be constructed by using reagents such as the 2-formylbenzeneboronic acid **1**, which, upon reaction with the amine group, would form a potentially stable iminoboronate, due to a well-known N−B interaction,[18−23] as depicted in Scheme 1.

Scheme 1. Lysine ε-Amino Group Modification Based on the Formation of Stable Imines with 2-Formylbenzeneboronic Acid



RESULTS

To test this hypothesis, we evaluated the reaction between the model 1-butylamine and 2-formylbenzeneboronic acid **1**. Very pleasingly, the iminoboronate **3** was formed at room temperature in 49% and 52% when the reaction proceeded in neat water or in a KPi buffer solution (50 mM, pH 8.02), respectively (Scheme 2). Variation of the reagents' stoichiom-

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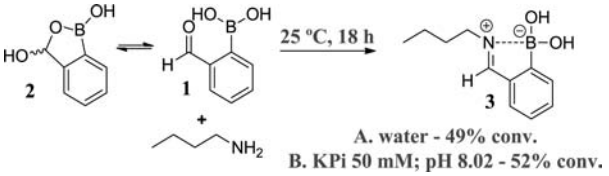
Scheme 2. Reaction of the Model 1-Butylamine and 2-Formylbenzeneboronic Acid in Neat Water and KPi Buffer Solution[a]

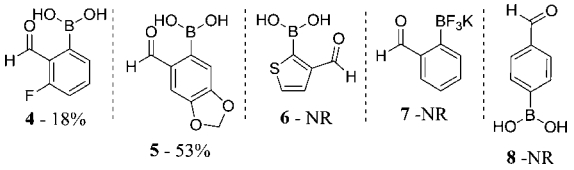


[a]Conversions determined on the basis of ^{1}H-NMR.

etry and dilution, buffer concentration, or reaction time had very little effect on the iminoboronate formation (see the Supporting Information).

Encouraged by these results, several formyl-boronic acids **4**–**8** were evaluated under these conditions, but none significantly improved the conversion obtained with **1**. The boronic acids **7** and **8**, which in principle cannot form a stabilized iminoboronate, did not react under these conditions (Scheme 3).

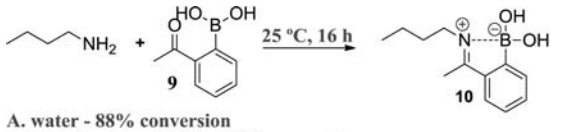
Scheme 3. Reaction of the Model 1-Butylamine and Formylboronic Acids 4–8 in KPi Buffer Solution (50 mM; pH 8.02); 18 h at 25 °C[a]



[a]Conversions determined on the basis of ^{1}H-NMR.

To reduce the impact of the competing mechanism that leads to the tautomeric compound **2** (see the Supporting Information),[24] we tested the use of stoichiometric amounts of 2-acetylphenylboronic acid **9**, and the imine was obtained in 88%, either in neat water or in KPi buffer solution (50 mM; pH 8.02). The reaction was quite fast, as only after 20 min at room temperature was the iminoboronate **10** formed in 81% (Scheme 4). The formation of the imine was also observed at pH values ranging from 6.01 up to 9.17. This linkage was shown to possess a remarkable stability, as, after 7 days under these conditions, the percentage of **10** remained unaltered (see the Supporting Information).

Scheme 4. Reaction of the Model 1-Butylamine and 2-Acetylphenylboronic Acid 9[a]



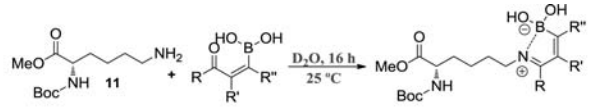
% of iminoborane **10**

pH	1 day	7 days
6.01	67%	67%
7.05	79%	80%
8.02	84%	88%
9.17	87%	87%

[a]Conversions determined on the basis of ^{1}H-NMR.

Iminoboronates generated from **1** or **9** are well-known for their ability to assemble complex macrocycles depending on the structure of the amine used.[25,26] Therefore, we tested the imine formation using lysine. As shown in Scheme 5, the reaction

Scheme 5. Reaction of the Protected Lysine 11 with Boronic Acids 1, 4, 5, and 9[a]



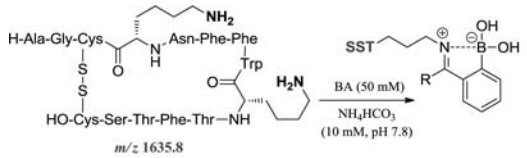
Boronic acid	Conv. %	Product	Boronic acid	Conv. %	Product
1	61%	11	5	56%	13
4	33%	12	9	71%	14

[a]Conversions determined on the basis of ^{1}H-NMR.

proceeded similarly to what was observed when using the model 1-butylamine, and again, the 2-acetylphenylboronic acid **9** proved to be the most efficient reagent, yielding the iminoboronate **14** in 71% (Scheme 5).

Taking these results into consideration, the functionalization of more complex biomolecules was attempted. Somatostatin (SST) is a short-life hormonal neuropeptide, which inhibits the secretion of various hormones, including the growth hormone. In tumor cells, membrane receptors for somatostatin are overexpressed, making it an excellent candidate to append and deliver cytotoxic agents.[27] The 14 amino acid active form of this peptide was employed as a target to test our hypothesis, since it has two exposed lysine residues (Scheme 6).[28] Therefore,

Scheme 6. Reaction of Somatostatin with Boronic Acids 1 and 9[a]



Conditions	+1 BA (*m/z*)	+2 BA (*m/z*)	+3 BA (*m/z*)
BA - **1** (50 mM)	1750.7 (-H_2O)	1863.8 (-2H_2O)	1996.8 (-2H_2O)
BA - **9** (50 mM)	1781.8	1927.8	-

[a]Conversions were determined on the basis of ESI-MS analysis; relative abundance with the most intense *m/z* peak.

somatostatin was mixed for 5 min with 2-acetylphenylboronic acid **9** (50 mM) in NH_4HCO_3 (10 mM, pH 7.8) and the reaction was analyzed by ESI-MS. Very gratifyingly, the conjugates with one and two modifications were readily obtained with almost complete conversion of the peptide. Similarly, though with slight less efficiency, the 2-formylbenzeneboronic acid **1** underwent conjugation with somatostatin, affording products with 1, 2, and 3 modifications (Scheme 6). Once the successful formation of the constructs was confirmed, MS^2 analysis of the conjugate with three modifications obtained with **1** (*m/z* 1996.8) was performed and lead to the

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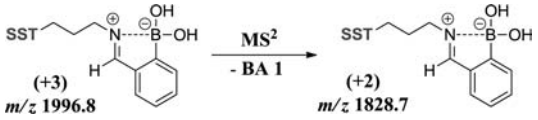
identification of a construct with one less appended boronic acid (Scheme 7).

Scheme 7. *m/z* Peaks Detected when a ESI-MS^2 Was Performed on a Modified Species of Somatostatin (*m/z* 1996.8)



Following the successful modification of somatostatin, the reaction was then attempted with lysozyme. As in the previous example, the modification took place smoothly at room temperature in $NH_4CH_3CO_2$ buffer solution (20 mM, pH 7.0) using 20 mM 2-acetylphenylboronic acid **9**. Remarkably, the reaction was quite tolerant to pH, and successful modifications were obtained in neat water or in buffer solution ($NH_4CH_3CO_2$, 20 mM) at pH values between 5.0 and 7.0 (Figure 1) (see the Supporting Information). These reactions

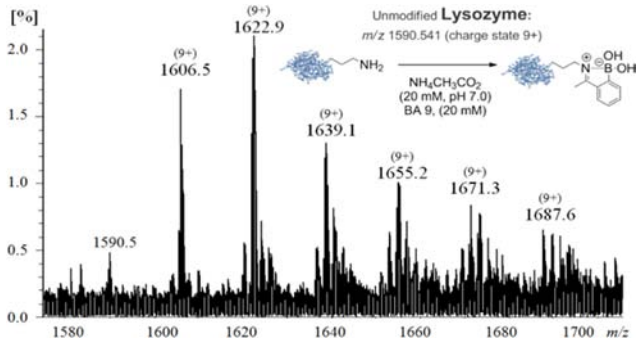


Figure 1. Reaction (30 min) of lysozyme (10 μM) with 2-acetylbenzeneboronic acid **9** (20 mM), at room temperature. Zoom of the (9+) charge state of the ESI-FTICR-MS spectrum.

were evaluated by performing native mass spectrometry, thus maintaining secondary and tertiary structures of the protein during the process of ionization (Figure 1).

Using the optimized conditions, lysozyme was reacted with boronic acids **1**, **4**, **5**, and **6**. Similarly to what was observed with 1-butylamine, no reaction was observed with boronic acid **6** (see the Supporting Information). Nevertheless, **1**, **4**, and **5** modified the protein, indicating the system tolerance toward different functionalities on the aromatic moiety (Figure 2). Finally, proteins such as cytochrome *c*, ribonuclease A, and myoglobin were also fully converted with 2-formylbenzeneboronic acid **1** in less than 5 min after addition of this boronic acid at room temperature (Figure 3).

Once the bioconjugation based on the iminoboronate formation was established, we conceived that the N−B dative bond could be disrupted by the influence of an external molecule, offering a mechanism to promote the imine hydrolyses and, by this way, to revert the modification performed on the protein.[29−31] Bioconjugation techniques that allow a selective modification of the protein and a stimuli-responsive unconjugation are currently very useful tools to design delivery systems such as antibody−drug conjugates that deliver cytotoxic drugs selectively to tumor cells.[32,33]

To test this possibility, a range of endogenous molecules (EM) was selected and evaluated in the hydrolysis of

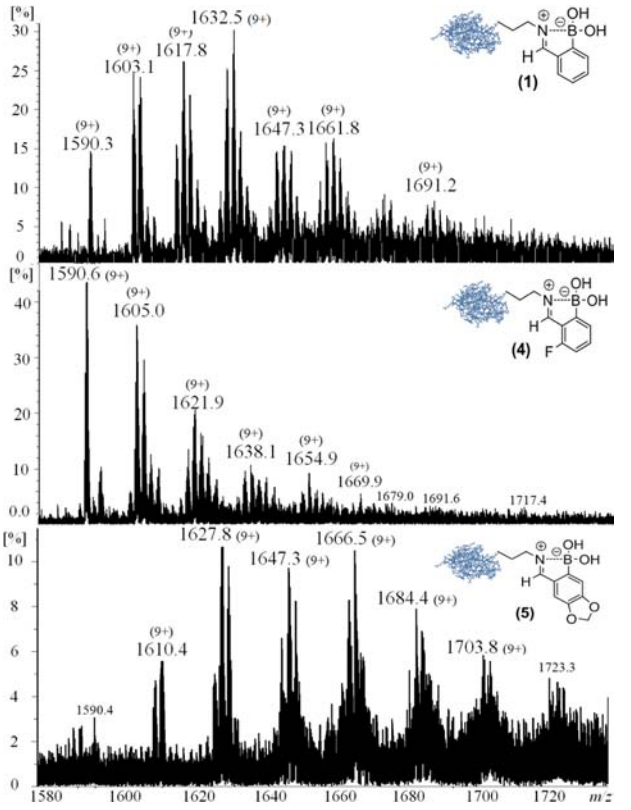


Figure 2. Reaction of lysozyme (10 μM) with boronic acids **1**, **4**, and **5** (10 mM). Zoom of the (9+) charge state of the ESI- FTICR-MS spectra.

iminoboronate **10**. As shown in Figure 4, most of the molecules tested had an almost negligible effect on the percentage of the preformed imine. Nevertheless, fructose and dopamine considerably reduced the concentration of **10** in 56% and 79%, respectively, probably due to the formation of boronate esters.[20−23,34] More importantly, glutathione decomposed the iminoboronate **10** in down to 7% in less than 2 h. This constitutes a very promising result when envisioning the design of delivery systems into cells because, in the cell cytoplasm, glutathione exists in an increased concentration (millimolar range) and this fact may be a target to promote the conjugate dissociation only when internalized.[35]

Taking into consideration the aforementioned results, the reversibility was evaluated using lysosyme as the model protein. With this goal in mind, the stability of the constructs formed between lysozyme and 2-formylphenylboronic acid **1** was first evaluated. Therefore, the conjugates were maintained at room temperature in a buffer solution ($NH_4CH_3CO_2$, 20 mM) at pH 7.0. By monitoring the reaction by ESI-FTICR-MS, it was clearly evident that the constructs maintained their integrity over a period of 5 h (see the Supporting Information)

On the basis of this, the hydrolysis potential was evaluated by the addition of dopamine (*a*), fructose (*b*), and glutathione (*c*). The ESI-FTICR-MS spectra displayed in Figure 5 were recorded immediately after the addition of 10 mM *a*, *b*, and *c* at room temperature. Similarly to what occurred when using the model iminoboronate **10**, we were delighted to observe that the addition of dopamine, fructose, and glutathione rapidly induced the hydrolysis of the constructs formed between lysozyme and **1**. More importantly, in the case of dopamine and

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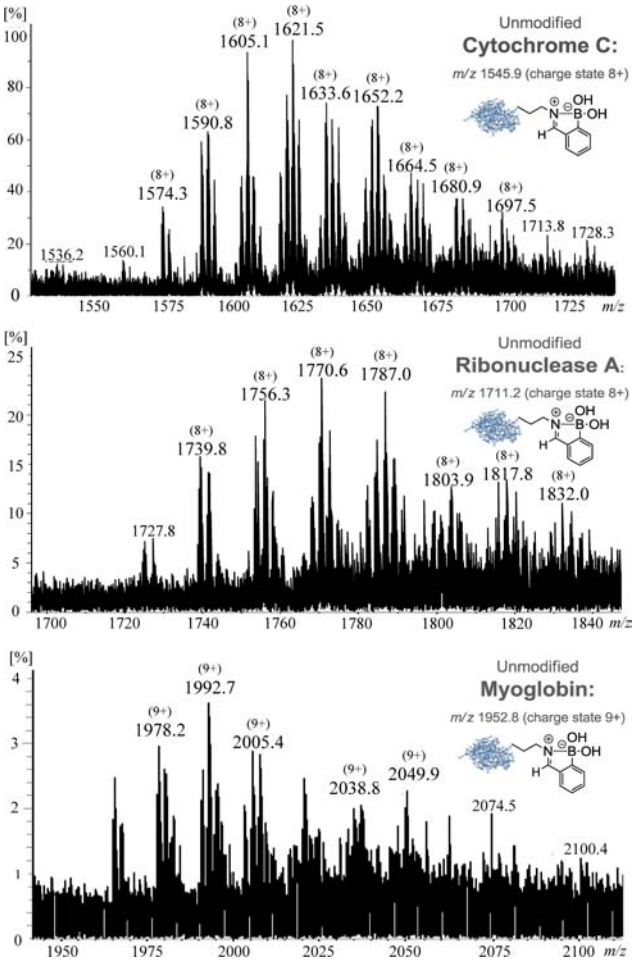

Figure 3. Reaction of cytochrome *c*, ribonuclease A, and myoglobin (10 μM) with 2-formylbenzeneboronic acid **1** (10 mM), at room temperature. Zoom of (8 or 9+) the charge state of the ESI- FTICR-MS spectra.

glutathione, the reversibility was not only fast but also complete under these conditions.

The mechanism of iminoboronate formation, from boronic acid **9** and 1-butylamine, was investigated by means of DFT

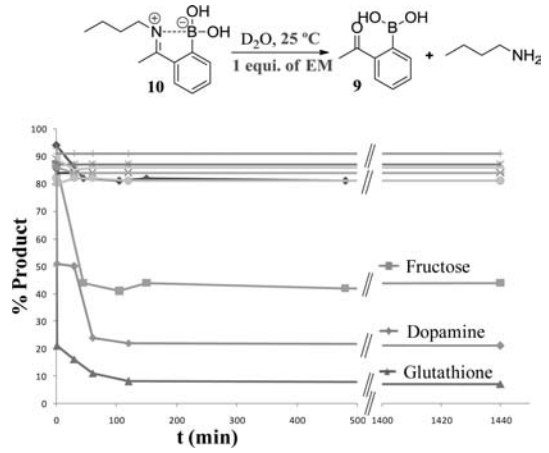

Figure 4. Reaction of iminoboronate **10** with 1 equiv of endogenous molecules (EM): glucose, fructose, lactose, sucrose, ephedrine, thymine, adenine, cytosine, dopamine, and glutathione.

calculations,[36] aiming at a deeper understanding of the remarkable stability of the product **10** toward hydrolysis and regeneration of the initial reactants. The energy profile obtained for that reaction is represented in Figure 6.

The mechanism of iminoboronate formation, represented in Figure 6, involves six consecutive steps starting with **A**, the pair of reactants, 1-butylamine, and boronic acid **9**, plus a water molecule. In the first step, from **A** to **B**, there is coordination of the O-atom of the carbonyl group to the boron (d_{B-O} = 1.63 Å), while the nitrogen approaches the carbonyl C-atom (d_{N-C} = 1.59 Å).

In the second step, from **B** to **C**, the nucleophilic attack from the nitrogen to the C-atom of the carbonyl group is accomplished with formation of the N−C bond and simultaneous protonation of the oxygen atom, resulting in hemiaminal **C**. The process of proton transfer is assisted by the neighbor water molecule, in such a way that one N−H proton is exchanged from the nitrogen of the amine to the water molecule, while, at the same time, one of the O−H protons of the solvent molecule is transferred to the O-atom of the carbonyl group in the boronic acid. In intermediate **C**, formation of the new C−N bond is finished, as shown by the corresponding distance (1.48 Å).

From **C** to **D** there is a rearrangement of the geometry of the pair hemiaminal-water and of the corresponding H-bond network. Then, in the following step, from **D** to **E**, there is $C-O_{OH}$ bond breaking, transfer of the hydroxyl group to the boron atom, and formation of an iminium ion. In the corresponding transition state, $\mathbf{TS_{DE}}$, this process is well advanced, with the former C−O bond practically cleaved (d_{C-O} = 2.26 Å) while formation of the new B−O bond is almost finished (d_{B-O} = 1.50 Å). Also, the N−C bond changes from a single bond in **D** (d_{N-C} = 1.48 Å), to a double bond in **E**, corresponding to the iminium $C{=}N^+$ bond (d_{N-C} = 1.29 Å).

The second proton of the nitrogen atom is transferred to one of the hydroxyl groups coordinated to the boron atom, in the fourth step of the path, from **E** to **F**. This process is assisted by the neighbor water molecule similarly to what happens in the second step of the mechanism. Thus, in **F**, boron coordination comprises the phenyl substituent, two hydroxyl groups, and one water molecule corresponding to the OH group in **E** that is protonated. This protonation results in a clear weakening of the corresponding B−O bond, as indicated by the corresponding bond lengths (1.50 Å, in **E**, and 1.64 Å, in **F**).

After another rearrangement of the H-bond involving the solvent molecule, from **F** to **G**, there is loss of a water molecule and formation of the iminoboronate molecule, in the fifth step of the mechanism (from **G** to **H**). In the corresponding transition step, $\mathbf{TS_{GH}}$, cleavage of the $B-O_{water}$ bond is finished, as shown by the corresponding distance (2.72 Å).

From **H** to **I**, there is a last reorganization of the H-bonds that, in this case, involves the iminoboronate molecule and two separated water molecules: the solvent molecule present in the model from the beginning of the path, and the one that corresponds to the reaction side product, formed in the previous step (from **G** to **H**).

In the last step of the mechanism, from **I** to **J**, there is an internal geometry adjustment in the iminoboronate molecule, corresponding mainly to a rotation around the NC−C(phenyl) bond. This process brings the imine N-atom and the boron to close proximity and allows the establishment of a B−N bond (d_{B-N} = 1.71 Å), greatly enhancing the stability of the product, **J**. In fact, the last step is strongly exoenergetic (ΔE = −7.4 kcal/

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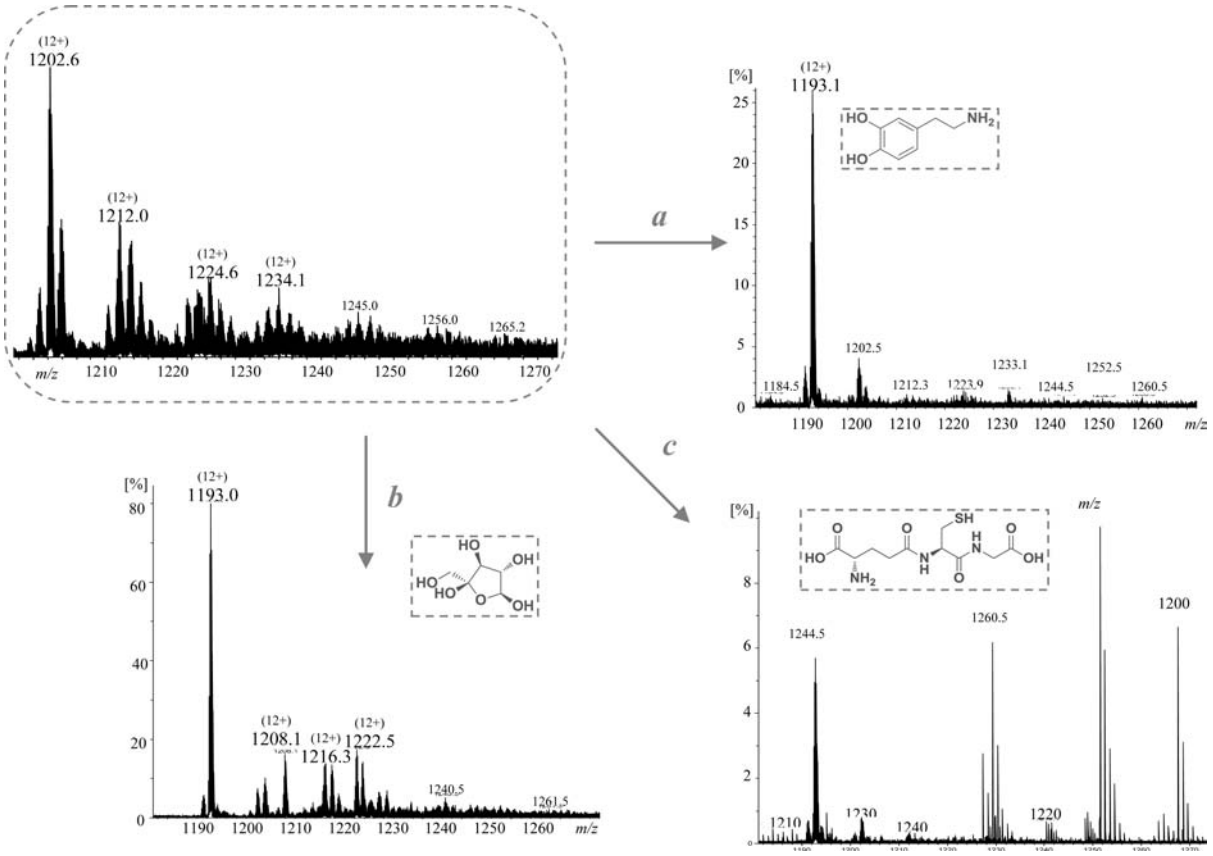

Figure 5. Reaction of lysozyme constructs with an equimolar amount of endogenous molecules (EM): dopamine, fructose, and glutathione. A zoom of the ESI-MS spectra of the lysozyme constructs is displayed. The ESI-FTICR-MS spectra *a*, *b*, and *c* were recorded immediately after the addition of dopamine, fructose, and glutathione, respectively.

mol), indicating the stability gain resulting from the formation of the B−N bond that is also reflected in an overall energy balance of −10.0 kcal/mol, indicative of favorable reaction, from the thermodynamic point of view.

The energy activation calculated for the reaction (12.4 kcal/mol) corresponds to the highest barrier along the path (from **D** to $\mathbf{TS_{GH}}$) being in good agreement with the experimental conditions employed, i.e., a reaction that occurs at room temperature. In fact, the most difficult steps along the entire path are the two proton transfer steps, from N−H to O−H. Despite the assistance of the solvent molecule in those processes, the corresponding transition states, $\mathbf{TS_{BC}}$ and $\mathbf{TS_{GH}}$, are both 5.9 kcal/mol above the reactants, **A**.

It is important to stress the role of the boron atom along the mechanism. The establishment of a B−O bond involving the oxygen atom originally from the carbonyl group starts by activating this group toward nucleophilic attack from the amine N-atom. However, the B−O bond is retained along most of the mechanism, from **B** to **G**, promoting the two protonation steps and, thus, assisting in the formation and loss of the water molecule that will be the leaving group in the reaction. This boron assistance along the reaction explains the significantly smaller energy barrier of the mechanism for the formation of the iminoboronate **10**, when compared to the equivalent reaction with a substrate without boron. In fact, the energy barrier calculated for imine formation from 1-butylamine and acetophenone is 35−36 kcal/mol (see Figures S87 and S88 of the Supporting Information).

However, the most important feature of the iminoboronates described in this work, in what concerns their potential use in protein modification, is their stability toward hydrolysis or, in other words, the diminished reversibility of the corresponding reaction when compared with simple imine formation. That stability is a direct consequence of the B−N bond established between the boron atom and the imine N-atom, as show by a 7.4 kcal/mol energy gain in the final product (**J**) compared with intermediate **I**, where the B−N bond is absent. In the optimized structure of **10** (Figure 7), the presence of the B−N bond is shown by a distance of 1.70 Å, and this is confirmed by a Wiberg index[37] of 0.48, indicative of a covalent bond.

Moreover, the *Z*-isomer of the iminoboronate (**H′**), where the stereochemical arrangement around the C═N bond prevents any B−N interaction, is 4.8 kcal/mol less stable than the initial reactants, **A** (see Figure S89 of the Supporting Information) and, hence, about 15 kcal/mol less stable than **J**. This indicates that formation of iminoboronates with a *Z*-arrangement around the C═N bond, such as **H′**, corresponds to a thermodynamically unfavorable reaction and, thus, is easily reversible.

The same happens for simple imine formation. The reactions where the ketone has no B-atom (acetophenone; see Figures S87 and S88 of the Supporting Information) are slightly endoenergetic (ΔE = 3−5 kcal/mol), indicating a reversible character that corresponds to the well-known tendency of imines to suffer hydrolysis.

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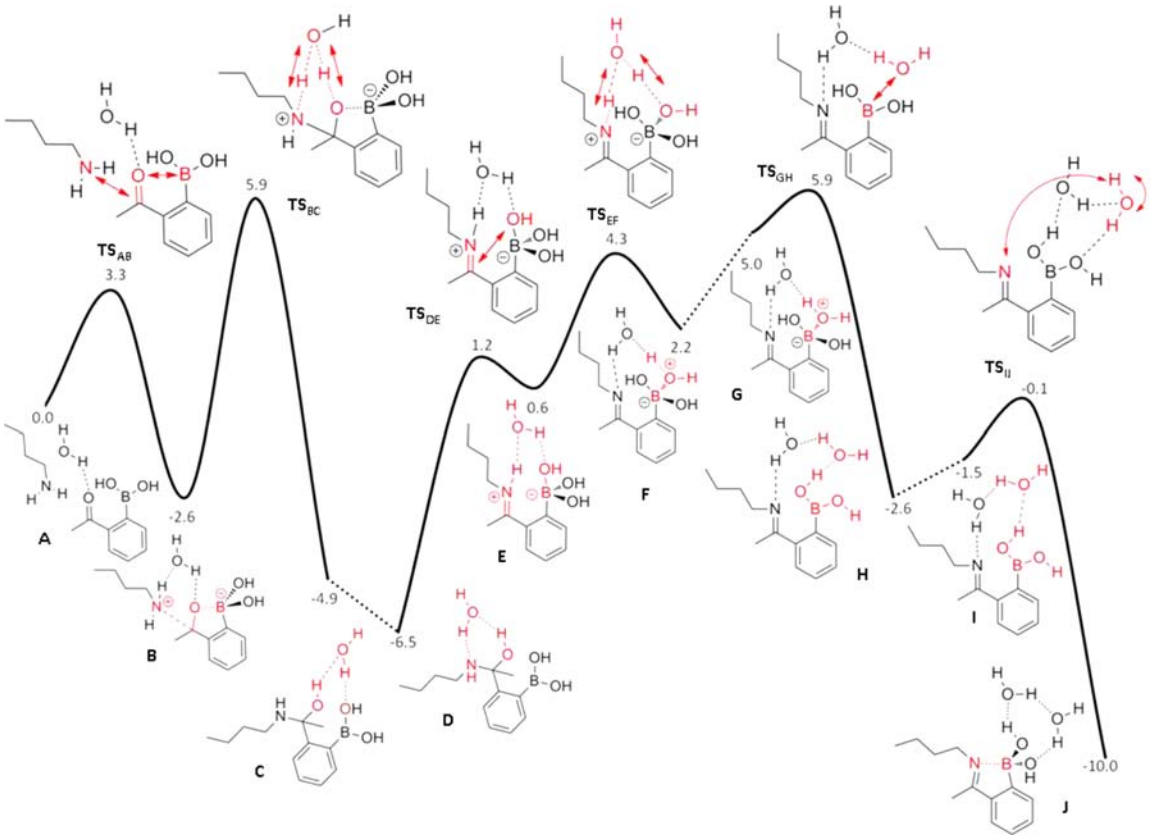

Figure 6. Energy profile (kcal/mol) calculated (PBE0/6-31G**) for iminoboronate (**10**) formation from 1-butylamine and acetylphenylboronic acid (**9**). Relevant changes, in each step, are highlighted in red.

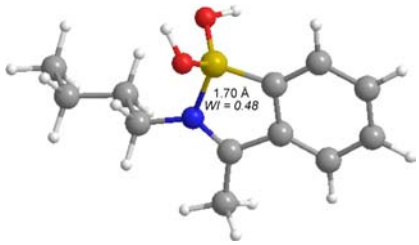

Figure 7. Optimized geometry (PBE0/6-31G**) of the iminoboronate **10**. The B−N bond distance (Å) and the corresponding Wiberg index (WI, italics) are indicated.

CONCLUSION

We presented here an innovative strategy to modify protein lysine ε-amino and *N*-terminal groups on the basis of the generation of stable imines in water with 2-carbonylbenzeneboronic acids. The formation of stable iminoboronates in water allowed the fast and efficient modification of somatostatin and model proteins such as lysozyme, cytochrome *c*, ribonuclease A, or myoglobin. More importantly, these reagents allowed an unprecedented mechanism by which the lysine may be unmodified upon addition of glutathione, dopamine, or fructose. A detailed DFT study was performed to rationalize the observed stability of the iminoboronate constructs. This study highlighted the importance of the boronic acid in the imine formation, and it revealed that the observed stability of this imine toward hydrolysis is most likely due to the formation of a dative N−B bond. We are currently developing new reagents for bioconjugation based on the iminoboronate strategy, and we plan to apply this innovative strategy to the synthesis of new reversible tumor-targeting anticancer drug conjugates and to the design of novel monosaccharide-responsive polymers for insulin delivery.

EXPERIMENTAL SECTION

General Procedure for Reactions with Somatostatin. 2-Formylbenzeneboronic and 2-acetylbenzeneboronic acids (50 mM) were reacted with 10 μM of somatostatin in NH_4HCO_3 buffer (10 mM, pH 7.8) at room temperature. After 5 min, an aliquot of the reaction mixtures was evaluated by ESI-MS.

General Procedure for Reactions with Lyzosyme. 2-Formylbenzeneboronic and 2-acetylbenzeneboronic acids (10 and 20 mM, respectively) were reacted with 10 μM of lyzosyme in $NH_4CH_3CO_2$ buffer (20 mM, pH7.0) at room temperature. After 30 min, an aliquot of the reaction mixtures was evaluated by ESI-FTICR-M. *Reactions with different proteins:* 2-formylbenzeneboronic acid (10 mM) was reacted with 10 μM of cytochrome *c*, ribonuclease A, or myoglobin in $NH_4CH_3CO_2$ buffer (20 mM, pH 7.0) at room temperature. After 5 min, an aliquot of the reaction mixture was evaluated by ESI-FTICR-MS.

Evaluation of the Reversibility. 2-Acetylbenzeneboronic acid (8 mg, 5.0×10^{-5} mol) was added to an eppendorf tube and then dissolved in 0.3 mL of D_2O. Afterward, 1 equiv of 1-butylamine (5 μL) was added to the same tube and these compounds were reacted for 16 h at 25 °C. Subsequently, a ^{1}H NMR spectrum was collected to evaluate the conjugation rate. Then, 0.1 mL of D_2O containing 1 equiv of different molecules was added. The reaction was then monitored by collecting ^{1}H NMR spectra. *Reversibility evaluation of lysosyme conjugates:* The reversibility of the linkage was evaluated by the addition of reduced glutathione, dopamine hydrochloride, and l-fructose (10 mM) to the modified lysosyme; the results were evaluated by ESI-FTICR-MS.

Computational Details. All calculations were performed using the Gaussian 03 software package, and the PBE0 functional, without

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symmetry constraints. That functional uses a hybrid generalized gradient approximation (GGA), including a 25% mixture of Hartree−Fock exchange with the DFT exchange-correlation, given by the Perdew, Burke, and Ernzerhof functional (PBE). The optimized geometries were obtained with a standard 6-31G(d,p) basis set. Transition state optimizations were performed with the Synchronous Transit-Guided Quasi-Newton Method (STQN) developed by Schlegel et al., after a thorough search of the potential energy surfaces (PES). Frequency calculations were performed to confirm the nature of the stationary points, yielding one imaginary frequency for the transition states and none for the minima. Each transition state was further confirmed by following its vibrational mode downhill on both sides and obtaining the minima presented on the energy profiles. A natural population analysis (NPA) and the resulting Wiberg indices were used to study the electronic structure and bonding of the optimized species.

The influence of basis set size and solvent on the calculated reaction mechanism was tested by means of single point energy calculations using a 6-311++G(d,p) basis set, and the geometries were optimized at the PBE0/6-31G(d,p) level. Solvent effects (water) were considered using the polarizable continuum model (PCM) initially devised by Tomasi and co-workers as implemented on Gaussian 03. The molecular cavity was based on the united atom topological model applied on UAHF radii, optimized for the HF/6-31G(d) level. The free energy values calculated with the better basis set and including solvent effects are similar to the electronic energy values obtained with the smaller basis set in the gas phase, and hence, these latter are the ones presented in the energy profile. For example, the activation free energy for the formation of **10** from **9** and 1-butylamine is 9 kcal/mol at the PBE0/6-311++G(d,p) (PCM)//PBE0/6-31G(d,p) level, compared to 12 kcal/mol, the energy barrier calculated at the PBE0/6-31G(d,p) level (cf. Figure 6). Similarly, the overall energy balance for the reaction is −7 and −10 kcal/mol, by the same order. The same happens when there is no B-atom involved in the reaction, i.e., the reagent is acetophenone (Figure S87), with a maximum difference of 2 kcal/mol for the relevant energy values calculated at the two levels of theory.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Experimental procedures, full DFT reference list, atomic coordinates for the optimized species, and spectral data for all compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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