Universidade de Lisboa

Faculdade de Farmácia

Targeting tumour cells usings chemical linkers with boronic acids and diazaborine scaffolds.

Ricardo Simões dos Santos

Trabalho de Campo orientado pelo Professor Doutor Pedro M. P. Góis, Professor Auxiliar com Agregação e coorientado pelo Doutor João P. M. António, Investigador.

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Ricardo Simões dos Santos

Trabalho Final de Mestrado Integrado em Ciências Farmacêuticas apresentado à Universidade de Lisboa através da Faculdade de Farmácia

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2022

Resumo

O cancro é uma doença cuja prevalência tem vindo aumentar ao longo dos anos e que na maioria dos casos leva à morte do individuo que a possui. A sua elevada taxa de mortalidade, assim como os custos que encarrega, levaram a indústria farmacêutica e a comunidade académica a juntar-se de modo a encontrar novas soluções terapêuticas. O uso de fármacos citotóxicos com elevada potência é uma das soluções. Contudo, estes possuem reduzida seletividade, podendo ser perigosos para o organismo. Foram então desenvolvidas terapêuticas alternativas para solucionar este problema.

Uma destas terapêuticas, é o uso de conjugados fármaco-anticorpo (ADCs). A estrutura dos ADCs está dividida em 4 componentes: um anticorpo, um fármaco citotóxico, um espaçador e um elemento de bioconjugação. As propriedades dos ADCs podem ser otimizadas alterando as estruturas dos seus componentes para que sejam mais eficazes e melhor tolerados em condições fisiológicas.

Outra terapêutica também do nosso interesse é a criação de aglomerados proteicos com capacidade de encapsulamento do fármaco e de o transportar até ao interior das células alvo libertando o mesmo no seu interior. Têm-se vindo então a estudar diferentes estruturas que consigam ligar duas proteínas entre si, mas que sejam facilmente degradadas no citoplasma da célula tumoral.

Para a realização deste projeto, tivemos como alicerce os ácidos borónicos (BAs), que são bastante versáteis devido à sua elevada reatividade. Estes permitem não só o desenvolvimento de ADCs, mas também de "linkers" inter-proteicos, devido à capacidade de se ligar a péptidos, assim como de reagir com hidrazinas para a formação de diazaborinas, moléculas estáveis em condições fisiológicas. Tivemos também em consideração a sua orbital *p* livre que permite reagir com espécies reativas de oxigénio (ROS) que se encontram em abundância em ambientes tumorais, e que levam à degradação destas moléculas. Durante este projeto focámo-nos então em planear e construir uma molécula bivalente que pudesse ser usada tanto como ADC assim como "linker" inter-proteico, que simultaneamente fosse sensível as condições fisiológicas especificas das células tumorais.

Palavras-chave: Ácidos borónicos (BAs); Cancro; Conjugados fármaco-anticorpo (ADCs); Fármacos citotóxicos; "linkers" inter-proteicos.

Abstract

Cancer is a disease whose prevalence has increased over the years and in most cases leads to the death of the individual who bears it. Its high mortality rate, as well as the costs involved in its maintenance have led the pharmaceutical industry and the academic community to come together in order to find new therapeutic solutions. The use of cytotoxic drugs with high potency is one of these solutions. However, the problem of these drugs is their reduced selectivity, which usually leads to the appearance of unwanted side effects. Different therapies were then developed to ameliorate this problem.

One of these therapies is the use of antibody-drug conjugates (ADCs). The structure of ADCs is divided into 4 components: an antibody, a drug, a spacer and a bioconjugation technology. These bioconjugates attempt to solve the problem of selectivity by relying on the antibody's high selectivity to deliver the payload safely to the intended site. ADCs properties can be optimized by changing the structures of their components so that they are as effective and tolerated as possible under physiological conditions.

Another promising therapy is the creation of protein clusters capable of encapsulating the drug and transporting it to the interior of the target cells and releasing the drug in the interior. Different structures have already been reported which are able to bind two proteins together, and that are easily degraded in the cytoplasm of the tumour cell.

On the idealization of this project, we considered boronic acids (BAs) as our foundation, as these molecules are quite versatile due to their high reactivity. They allow not only the development of ADCs, but also inter-protein linkers, due to their ability to bind with peptides, as well as react with hydrazines to form diazaborines, molecules that are stable under physiological conditions. We also took into account its free *p* orbital, which to reacts with reactive oxygen species (ROS) that are found in abundance in tumour environments, and lead to the degradation of BA´s. During this project, we focused on planning and building a bivalent molecule that could be used both as an ADC linker as well as an inter-protein linker, which was simultaneously sensitive to the specific physiological conditions of the tumour cells.

Keywords: Boronic acids (BA's); Cancer; Cytotoxic drugs; Drug-antibody conjugates (ADC's); Inter-protein linkers.

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Em primeiro lugar, quero agradecer ao Professor Doutor Pedro Góis, por ter visto em mim o potencial necessário e de meter dado a oportunidade de poder trabalhar sob a sua orientação durante os passados 18 meses nesta área que me é tão querida. Agradecer também a sua proposta continua de novos desafios e de novas descobertas, que me motivavam a continuar o meu trabalho e que me fizeram chegar aqui com uma mala cheia de novas experiências e vivências. Deixar também um agradecimento ao Doutor João António, por toda a paciência, simpatia e por toda a ajuda que me ofereceu nesta fase final do meu projeto, assim como à Doutora Alba Blázquez por ter sido a primeira a me ajudar a dar os primeiros passos neste mundo que é a investigação.

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Abbreviations

^{1}H NMR- proton nuclear magnetic resonance

2-ABBA- 2-acetylbenzeneboronic acid

2-CBBA- 2-carbonylbenzeneboronic acid

2-FBBA- 2-formylbenzeneboronic acid

^{13}C NMR- carbon nuclear magnetic resonance

AcONa- sodium acetate

ADC- antibody-drug conjugate

Ar- Argon

B_2pin_2- bis(pinacolato)diboron

BA- boronic acid

BE- boronate ester

BSA- bovine serum albumin

Chloroform-*d*- deuterated chloroform

DAB- diazaborine

DCM- dichloromethane

DMF- dimethylformamide

Et_3N- triethylamine

EtOAc- ethyl acetate

GSH- glutathione

H_2O_2- hydrogen peroxyde

LRMS- low resolution mass spectrometry

NHS- *N*-hydroxysuccinimide

Oxi-DEX- oxidation-sensitive dextran

PC1- Peroxy Crimson 1

[$Pd(dppf)Cl_2$]- [1,1'-bis(diphenylphosphino)ferrocene]palladium (II) chloride]

PFA- paraformaldehyde

PG1- Peroxy Green 1

$PhN(Tf)_2$- *N*-phenyl-bis(trifluoromethanesulfonimide)

ROS- reactive oxygen species

SDS-PAGE- sodium dodecyl sulphate–polyacrylamide gel electrophoresis

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1 Introduction

1.1 Boronic Acids

In the list of relevant elements in synthetic organic chemistry, there is no doubt that boron is one of the most useful ones. Not only is this element the fifth of the periodic table occupying privileged position, boron also is considered a metalloid incorporating characteristics of both metals and non-metals. Boron has a vacant *p* orbital that can reversibly interact with nucleophiles, shifting from a neutral trigonal planar structure to a tetrahedral boronate, which gives this atom such interesting chemical properties. (1,2)

Boron is a very abundant element and can be found in its mineral form such as borax and kernite (Figure 1). Despite the fact that when found in their natural sources the boron appears as boric acids, it is known that C–B bonds can be achieved by synthetic methods. This C-B bonds opens the way to a number of different molecules that can have very interesting biological properties. Boronic acids and diazaborines are two of these chemical structures that incorporate this C-B bond, and that we will work with. (3)

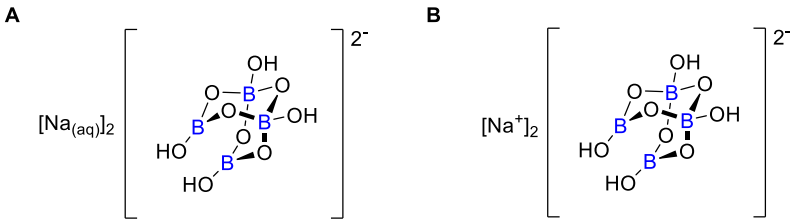


Figure 1. Structure of boron found in its natural form, A) borax and B) kernite.

In light of this discoveries made around the boron atom, medicinal chemists started developing new boronated molecules with improved biological activity. As mentioned before, one of these molecules are the boronic acids. These chemical structures are trivalent organic compounds that have a C-B bond as well as two hydroxyl groups bonded with the boron. In boronic acids, the boron atom adopts a trigonal planar geometry and possesses six valence electrons placed on *sp2*-orbitals as well as a vacant *p*-orbital. (4)

Chemically, these functional groups have unique properties and reactivity as mild Lewis acids, which means that they are uncharged at physiologic conditions. These

properties offer BAs a fairly good stability in physiological environments. (4) BAs were also proved to be able to form very interesting interactions with different ligands, such as diols and amines, to form boronate esters and iminoboronates (Figure 2). These dynamic structures can be further applied in protein modification and synthesis of therapeutic bioconjugates. (5)

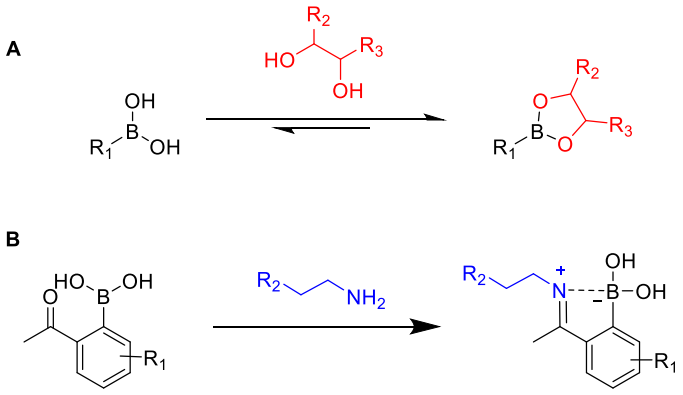


Figure 2. Interaction between boronic acid with, A) a diol to form a boronate ester and B) an amine to form an iminoboronate.

In the case of these bioconjugates, it is possible to use boronic acids to synthetize reversible linkers that are responsive either to acid environment, glutathione (GSH) as well as reactive oxygen species (ROS), properties that make them one of the fundamental structures of our entire project. (6)

1.1.1 Boronate Esters

Due to its promiscuous coordinative ability, BAs can be quite difficult to handle when the hydroxyl groups are not masked. Therefore, the replacement of the hydroxyl groups with alkoxy or aryloxy groups is a viable alternative to improve their handling. This replacement allows the formation of boronate esters (BEs) (Figure 3). Boronate esters are less polar and easier to handle, due to the loss of the hydrogen bond donor capability of the hydroxyl group of the BA, (7) a property that we took in account in our project, since we used a boronated pinacol to increase the stability of our molecule. The advantageous properties of BEs aroused the interest of chemists, who began to study the applicability of these molecules in bioconjugation strategies, both with natural and artificial residues.

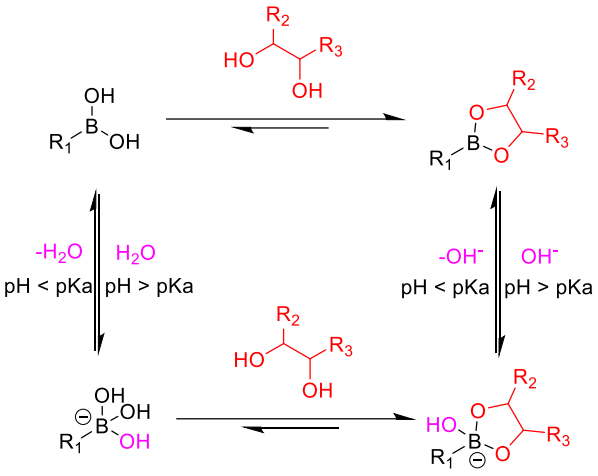


Figure 3. pH-Dependent formation equilibrium of a boronic ester in aqueous

Regarding the bioconjugation with natural residues, Markussen and co-workers were able to develop a delivery system for insulin that is sensitive to the increasing concentration of *D*-sorbitol. They prepared this system by functionalizing insulin in a lysine residue with a molecule that displayed both a BA and a polyol that, upon interaction, generate an inactive multiprotein complex (Figure 4). (8) These structures demonstrated an improved circulating half-life. However, in the presence of high concentrations of *D*-sorbitol they were degraded releasing insulin monomers. As for the bioconjugation with artificial residues, in 2008, Schultz group performed a site-specific incorporation of a non-natural boron-based amino acid into proteins using the amber stop codon strategy. The obtained modified protein was able to form BEs with poly-hydroxylated compounds that allowed a one-step traceless protein purification. (9)

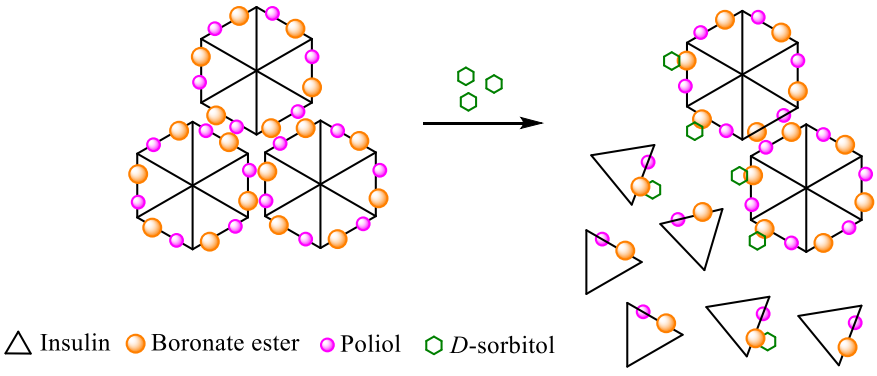


Figure 4. Insulin self-assembly under carbohydrate control.

1.1.2 Iminoboronates and Thiazolidines

As mentioned before, BAs are able to react with different ligands yielding an interesting range of different interactions. (5) The coordination between functionalized boronic acids and amino acids is one of these examples. Góis and co-workers were able to prove that compounds containing a boronic acid *ortho* to a carbonyl group, were able to react with lysine and cysteine residues of proteins to form functional bioconjugates with interesting biological activities. (5,10,11)

Regarding the coupling with lysine residues, in 2012, Góis group proved that the reaction between 2-formylbenzeneboronic acid (2-FBBA) and an *N*-terminal lysine formed a stable iminoboronate (Figure 5). (10) They were able to efficiently modify somatostatin under aqueous conditions, as well as other model proteins such as lysozyme, cytochrome c, ribonuclease A, or myoglobin. They also proved that the reaction with lysines also occurred when using 2-acetylbenzenoboronic acid (2-ABBA). (11) In this case, the reactions not only take place at the *N*-terminal lysine, but also with lysines that are incorporated in the middle of the amino acid chain. (12) This reaction yields an alkylic iminoboronate, reversible in the presence of fructose, dopamine, and GSH, via disruption of the bond between the boron and nitrogen atom.

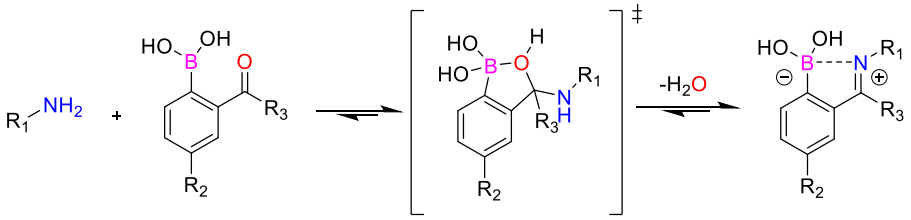


Figure 5. Iminoboronate formation and reversibility in aqueous media.

As for the coupling with the *N*-terminal cysteine residues, Góis and Gao groups described independently that the interaction between an *N*-terminal cysteine and 2-FBBA led to the formation of a boronated thiazolidine (Figure 6). (5,13) They proved that the initial interaction between the boron and the nitrogen atoms afforded a reactive imine that swiftly suffers an intramolecular thiol addition yielding the final thiazolidine. This interaction between the boronic acid and the *N*-terminal cysteine proved to be one of the fastest reactions for protein labelling.

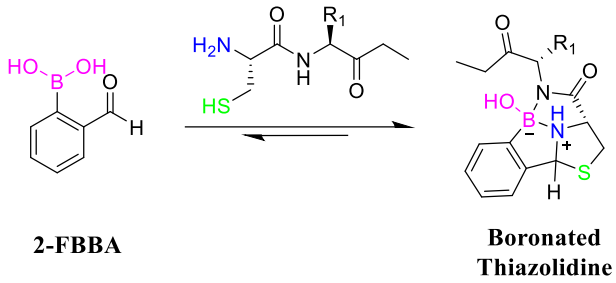


Figure 6. Formation of a boronated thiazolidine via modification of the *N*-terminal cysteine with 2-formylbenzeneboronic acid.

1.1.2.1 Reversibility of the iminoboronates

During the long years of study on cancer, several characteristics common in most tumour environments have been discovered, some of them being quite interesting for the implementation of these new therapies with drug delivery systems. Two of these features are the increased intracellular concentration of reactive oxygen species, mainly due to the mutations in mitochondrial enzymes, and elevated levels of glutathione. (14,15)

1.1.2.1.1 Oxidation with ROS

Reactive oxygen species are a group of molecules that include superoxide anion ($O_2^{\bullet -}$), hydrogen peroxide (H_2O_2), hydroxyl radical ($OH^{\bullet}$), singlet oxygen ($^1O_2$), peroxyl radical ($LOO^{\bullet}$), alkoxyl radical ($LO^{\bullet}$), lipid hydroperoxide (LOOH), peroxynitrite ($ONOO^-$), hypochlorous acid (HOCl), and ozone (O_3), among other molecules. (16) Simon and co-workers were able to convert arylboronic acids to the respective phenols simply adding a solution of H_2O_2 (mostly used as a model to ROS) to different substituted arylboronic acids, obtaining the respective phenols with good yields. (17) Some years later, in 2021, Dong's group proved that these conversions could be performed at room temperature within a very short reaction time. (18) They were able to obtain yields up to 99% of phenol with only 1-5 min of reaction. (18) This conversion follows a simple mechanism (Figure 7).

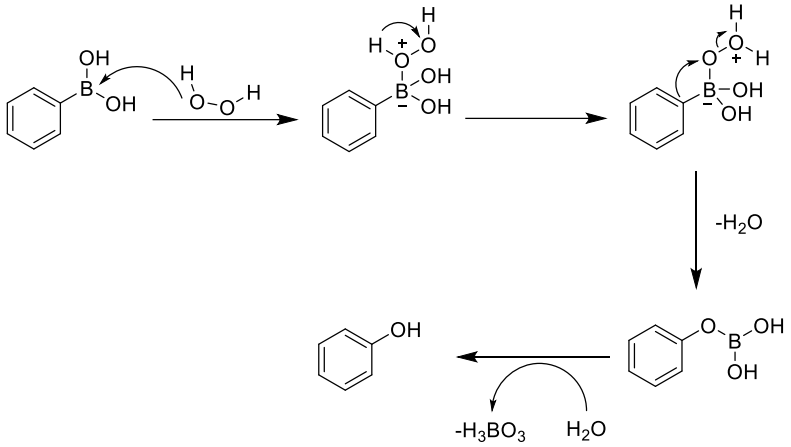


Figure 7. Conversion mechanism of arylboronic acids to phenols

The easy conversion of arylboronic acid/ester groups to their respective alcohol allowed the development and design of ROS-sensitive groups with various applications, such as method for detection and measurement of H_2O_2 in physiological conditions. (19) In 2007, Miller and co-workers prepared two probes, monoboronated 2-methyl-4-methoxy Tokyo Green (Peroxy Green 1, PG1) and monoboronated resorufin (Peroxy Crimson 1, PC1), both containing an arylboronic ester and reacted them in H_2O_2. They realized that the probes were easily oxidized to obtain 2-methyl-4-*O*-methyl Tokyo Green and resorufin, respectively, which are molecules that are fluorescent (Figure 8). These results proved that these probes are very useful for the measurement of the levels of hydrogen peroxide in the cells. (20)

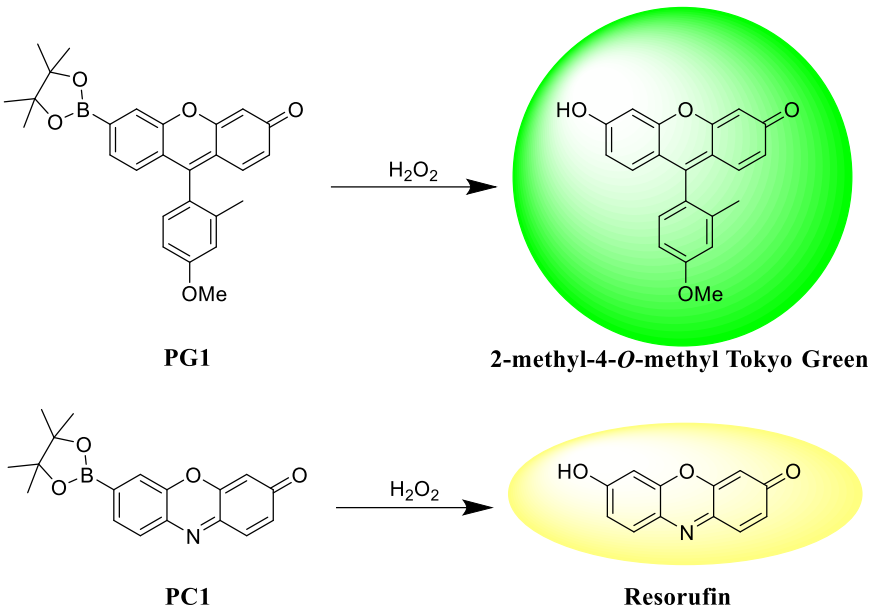


Figure 8. Activation of the nonfluorescent probes PG1 and PC1 with hydrogen peroxide.

Another one of these applications is based on a solubility switch strategy via H_2O_2 oxidation. (21) In 2011, Broaders group was able to modify the hydroxyls of dextran, which was very soluble in water, with arylboronic ester groups obtaining a water insoluble oxidation-sensitive dextran (Oxi-DEX). They proved that in the presence of H_2O_2 the Oxi-DEX polymers suffered oxidation of boronic esters, leading to the exposure of the hydroxyl groups and subsequent polymer degradation. (22)

1.1.2.1.2 Reduction with GSH

GSH is a molecule that can usually be found in the interior of cells and has been demonstrated to have specific biological roles *in vivo*. (15) The intercellular concentrations of these molecules are a very good indicator for the cellular oxidative stress level and can be directly associated with a cellular malfunction. It was studied the levels of GSH inside tumour cells, and compared to the intercellular concentration of this molecule in normal cells, the levels are 1.7–7 fold higher in the tumour cells. (15)

The high concentrations of GSH inside the cancer cells could be explored in the preparation of GSH-responsive linkers. (23) Hao and co-workers demonstrated that they could synthetize iminoboronate-forming prodrugs for in-situ albumin binding.

They also proved that the same iminoboronates were degraded and released the loaded drugs when exposed to intracellular GSH (Figure 9). (24) Furthermore, Gois group, was also able to design a targeting conjugate that was obtained *via* a three-component reaction between BAs, aminophenols and salicyl aldehydes. (6) Although these new conjugates were very stable in physiological conditions, they were also swiftly hydrolysed in the presence of high concentrations of GSH.

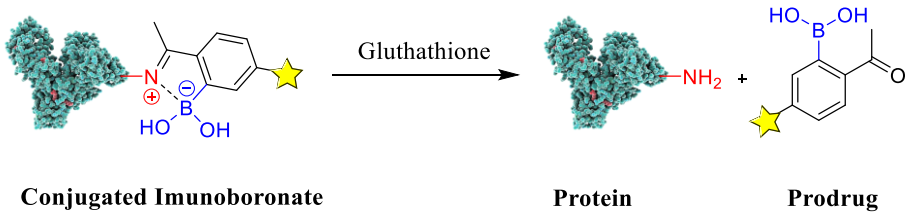


Figure 9. Reduction of an iminoboronate releasing a produg when exposed to high concentrations of GSH.

1.1.3 Diazaborines

We have already seen that BAs can react fairly easy endogenous molecules, such as ROS and GSH, granting them a relatively low stability under physiological conditions. Regarding this, some studies have been made to find out an alternative that would retain the interesting boronic acid reactivity but that could have a better stability when used in therapeutics, and diazaborines (DABs) contained all these features. (25)

DABs are compounds that are included in the class of the boron-nitrogen heterocycles, which can be synthesized by the reaction between a boronic acid and an hydrazine. (26) These molecules were firstly reported in the 60's (26) were used in therapeutics as antibiotics, due to their capability of inhibiting the lipopolysaccharide biosynthesis (27) and the inhibition of the enoyl reductase. (28,29) DABs are also very interesting compounds due to their electronic properties. The covalent bond between the boron atom and the nitrogen is able to form a stable zwitterion due to the capability of the nitrogen to donate its pair of electrons to the boron p-orbital, as well as the high stability it provides to the molecule. (30)

In the beginning, the synthesis of these molecules was achieved using very harsh conditions, such as very high temperatures. (26) Kanichar and co-workers tried to combine different *ortho*-carbonyl substituted phenylboronic acids with various

carbohydrazides under very high temperatures. (31) They used a total of 23 different carbohydrazides and tried to find out what products they would obtain by performing these combinations. They concluded that the product was different depending on the reaction conditions that were used (aqueous ethanol at room temperature or refluxing acetonitrile, as well as differences in the pH). From all the carbohydrazides tested, 20% yielded a hydrazone. Only a few reactions resulted in 2-acylated-1,2-dihydro-1-hydroxy-2,3,1-benzodiazaborines. The remaining of the reactions performed yielded anhydrous dimers of the DABs as the product. (31)

It was in 2015, that Susane Bane and her co-workers completely revolutionized the way of synthesizing DABs, performing a reaction under neutral aqueous conditions. (32) This synthesis was based on the combination of 2-formylphenylboronic acid with 4-hydrazinylbenzoic acid in aqueous conditions. In the end, they were able to obtain a single stable product, the 1,2-dihydro-1-hydroxy-2,3,1-benzodiazaborine. This DAB proved to be stable in solution for over a month (Figure 10). (32)

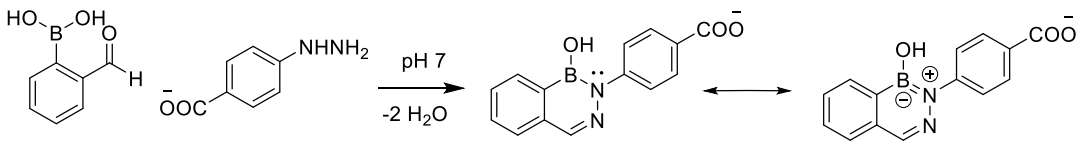


Figure 10. Synthesis of the 1,2-dihydro-1-hydroxy-2,3,1-benzodiazaborine using 2-formylphenylboronic acid and 4-hydrazinylbenzoic acid as reactants.

The toxic properties of the phenylhydrazine prompted the search for an alternative hydrazine that would be safer and easier to handle, in order to be employed in bioconjugation applications. In 2017, Gao's group proposed the synthesis of a DAB using a semicarbazide instead of a phenylhydrazine reacting with 2-carbonylbenzeneboronic acid (2-CBBA). (33) This reaction between the semicarbazide and the boronic acid gave results as good as the previous ones and with a similar formation rate, as well as a DAB that was very stable under biological conditions. (33)

1.1.3.1 Diazaborines in bioconjugates

Due to DAB's very interesting properties, such as high formation rates, under biocompatible reactional conditions, and better stability compared to the BAs, these

molecules started to be seen as a very viable way to achieve an innovating path on the bioconjugate engineering process.

Bane and co-workers were one of the first groups to try this protein labeling technique, in which a DAB was used as a linker between a fluorescent probe and bovine serum albumin (BSA). (32) In the synthesis of this new bioconjugate it was used BSA, that was previously modified with *N*-hydroxysuccinimide (NHS) ester to obtain a BSA-phenylhydrazine. This modified BSA was reacted with a 2-FBBA-coumarin complex in a phosphate buffer at pH 7 at room temperature (Figure 11). The product was then analyzed by fluorescence spectroscopy and sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) and the results proved that the bioconjugate was formed, due to the presence of fluorescence in the target protein. This synthesis proved that not only DABs are a viable option as a linker in bioconjugates but also their high stability under harsh conditions of analysis, such as the low pH of the SDS-PAGE resolving gel. (32)

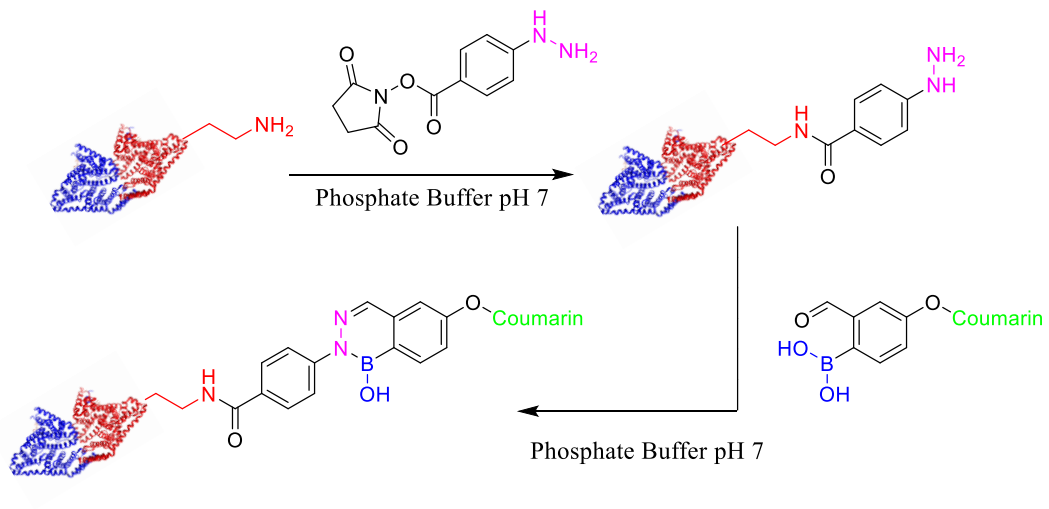


Figure 11. BSA functionalization with a fluorescent coumarin probe using a DAB linker under biocompatible conditions.

There was another discovery regarding the association of a fluorescent probe with a protein using a DAB as a linker. Gao's group found out that the continuous and dynamic rearrange of the bacterial peptidoglycan allowed the incorporation of a 2-ABBA. (33) After the 2-ABBA amino acid's incorporation in the peptidoglycan, the bacteria were incubated with a fluorescein-semicarbazide to allow the formation of the DAB and the label of the peptidoglycan with the fluorescent probe (Figure 12). The bacteria were then analysed via fluorescent microscopy and flow cytometry. The results showed that

the majority of the fluorescence was located in the cell envelope, which is the place where the bacterial peptidoglycan is found, proving that this incorporation was possible and a viable process to accomplish. (33)

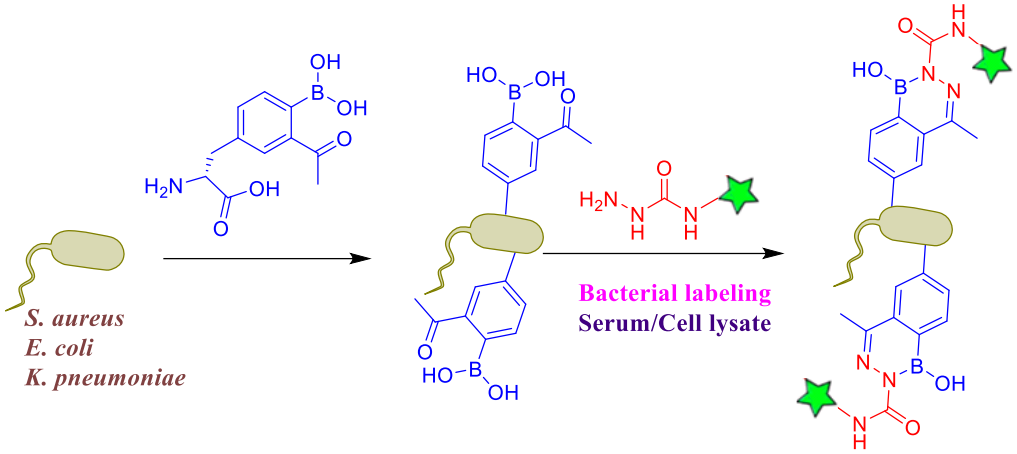


Figure 12. Schematic process of the incorporation and labelling of the 2-ABBA amino acid with a fluorescent probe in the bacterial peptidoglycan.

2 Objectives

The main objective of this project is to synthetise a molecule [1-(5-(hydroxymethyl)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)ethan-1-one] containing a boronic acid *ortho* to a carbonyl group and a *meta* hydroxymethyl. We also want to test if this molecule can be used as an inter-protein linker, by forming two iminoboronate when reacted with two *N*-terminal lysines of two proteins, and as a part of an ADC linker scaffold, by forming a diazaborine when reacted with a hydrazine containing a payload (fluorescent probe or cytotoxic drug) (Figure 13).

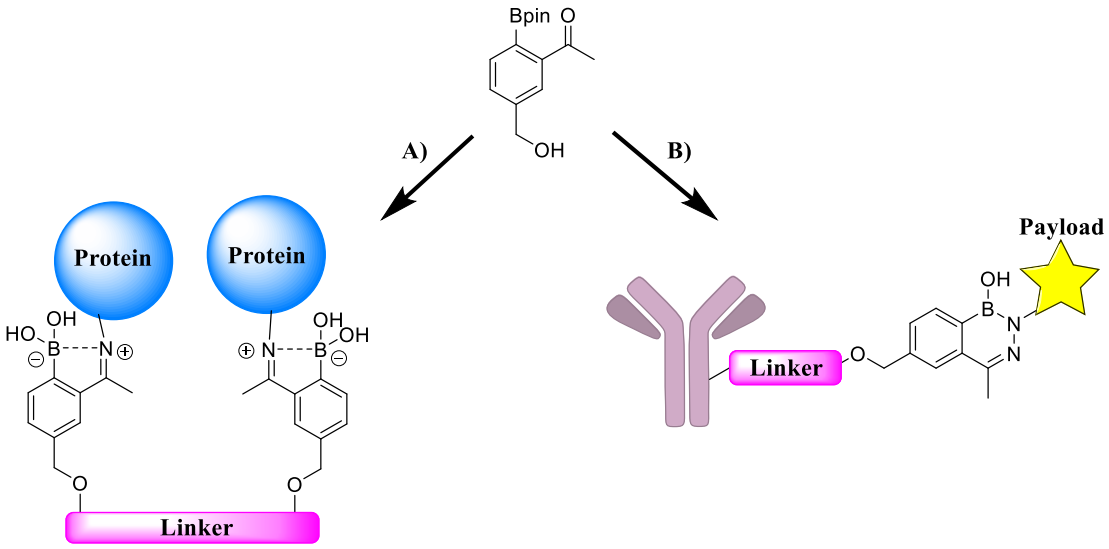


Figure 13. 1-(5-(hydroxymethyl)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)ethan-1-one as part of, A) inter-protein linker; B) ADC linker.

In this work we will describe the synthetic pathway to obtain the desired molecule, which we will react with a linker to try to obtain the inter-protein linker. We will also describe the reaction between our molecule and a hydrazine to verify if the formation of a diazaborine occurs.

3 Results and Discussion

3.1 Boronic acid and inter-protein linker

In the past years, it was showed that 2-carbonyl-benzenoboronic acids (2-CBBA) interacted with exposed lysine residues via the formation of iminoboronates. (11) The boronic acid *ortho* to a carbonyl is key for the success of this iminoboronate formation as it promotes the formation of an imine that is stabilized the by a N–B dative bond. (11) Based on this, we idealized a molecule that, not only had the ability to form a linkage with a lysine residue, but that could also do it with two of these residues. This molecule (compound **8**) was designed to serve as a linker between two proteins, and can be obtained via two sets of reactions that converge into one last reaction that yields the final product (Figure 14).

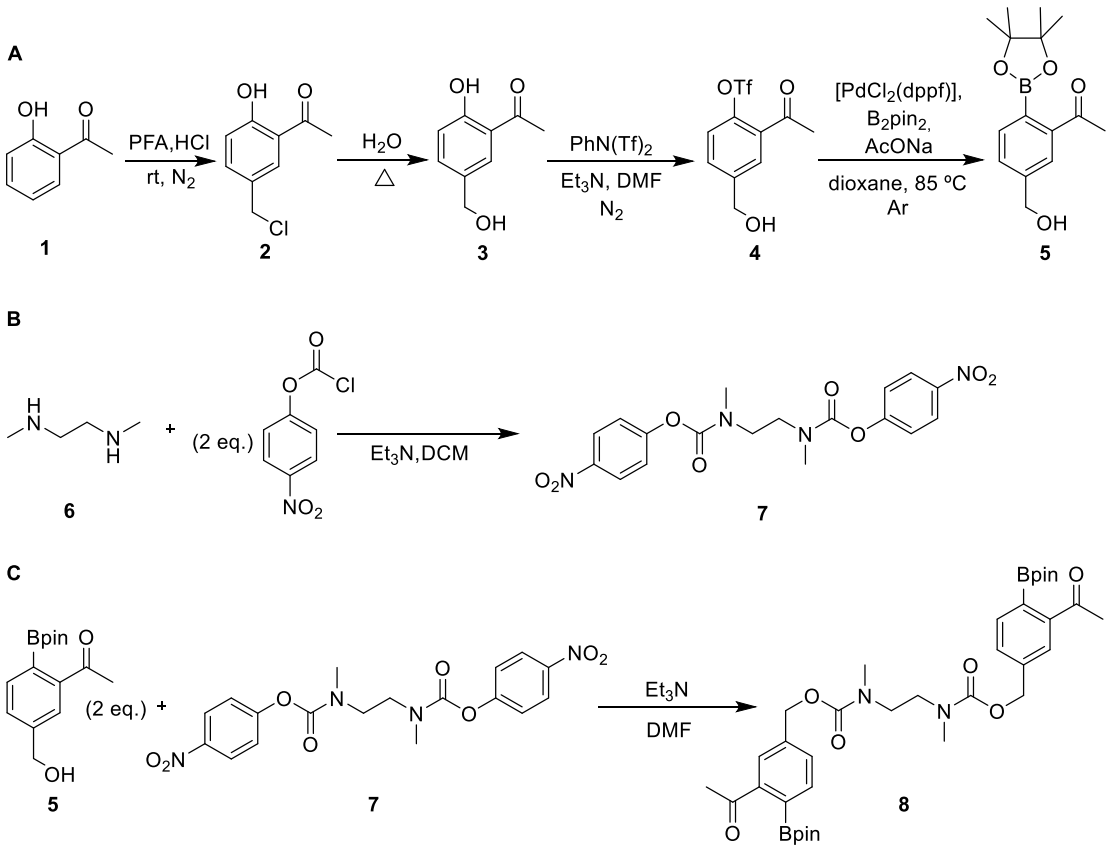


Figura 14. Synthetic pathways to obtain, A) compound 5, B) compound 7 and C) compound 8.

The functionalized boronic acid as well as the carbonyl group is the key factor to the whole project. One of the first goals, and the most important to the whole project, was

to obtain compound **5** which contains a boronate ester of pinacol *ortho* to a carbonyl. The first reaction that we performed was the addition of the hydroxymethyl substituent, *meta* to the carbonyl, to compound **1**. This hydroxymethyl group plays a major role, being this group that will further ahead react with the carbamate group of compounds **7** to yield compound **8**. The reaction to obtain compound **3** is divided in two different reactions that have compound **2** as an intermediary, which was not isolated. In this synthetic method, we firstly have to insert a chloromethyl *meta* to the carbonyl (Figure 15) followed by substitution of the chlorine by a hydroxyl group to obtain the desired hydroxymethyl substituent.

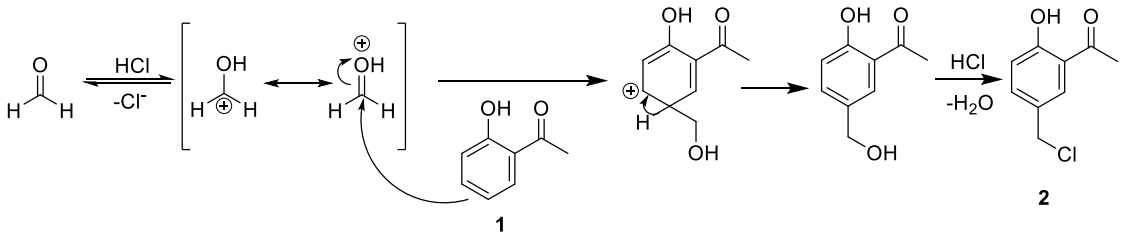


Figura 15. Compound 2 synthetic mechanism.

The next step was to transform the *ortho* hydroxyl group of compound **3** to a boronic acid. The easiest and most common way to obtain this boronic acid in the molecule is via a Miyaura borylation. (34) However, this reaction cannot immediately occur if the reagent has the hydroxyl group, due to the fact that this reaction requires a halogen or a triflate substituents, which are great leaving groups.

In order to perform the Miyaura borylation, we first needed to convert compound **3** to its triflated equivalent. For that to happen, we reacted compound **3** with *N*-phenyl-bis(trifluoromethanesulfonimide) [$PhN(Tf)_2$] in dimethylformamide (DMF) (Figure 16). We used $PhN(Tf)_2$ because it is milder, and therefore more selective, than most of the other triflate reactants, reacting only with the *ortho* hydroxyl group and not with the *meta* hydroxymethyl. We had to perform this reaction several times, always changing some reactional condition (equivalents of triethylamine used) or workup step (number of washes with brine in the liquid/liquid extraction), in order to optimize it. We ended up being able to improve the reaction yield from 36% to 49%, and to isolate the desired compound with no impurities, which in the first attempts was not possible (we were obtaining a mixture of compound **3** with the desired compound that could not be separated by column chromatography).

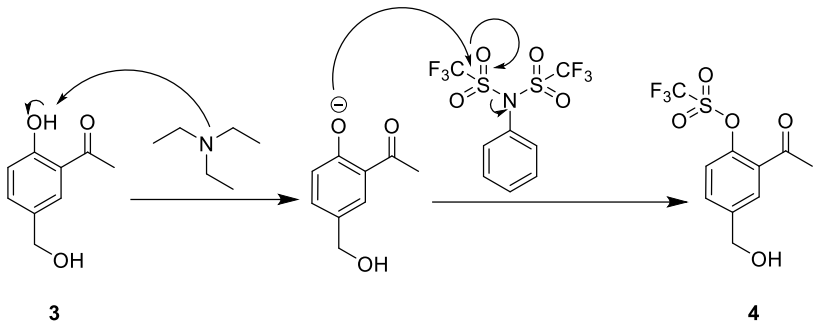


Figure 16. Compound 4 synthetic mechanism.

After obtaining compound **4**, we could now insert the boronic acid, that will allow the formation of bioconjugates through an iminoboronate ligation. The process of borylating a compound via a Miyaura borylation is very tricky and must be done with precision in order for the reaction to occur with no setbacks. (12) We had to conduct the reaction under argon atmosphere and a temperature of 85 ºC using previously dried dioxane during 12h. The duration is a very important reactional condition, because, if the reaction is left for too long, a protodeboronation process might occur, leading to the irreversible formation 1-(3-(hydroxymethyl)phenyl)ethan-1-one. So, we reacted compound **4** with [1,1'-bis(diphenylphosphino)ferrocene]palladium (II) chloride, bis(pinacolato)diboron and sodium acetate under the reactional conditions previously described in the literature. (12) This reaction occurred via a complex mechanism (Figure 17), and after purifying the crude we collected our desired product with a 63% yield as an orange oil.

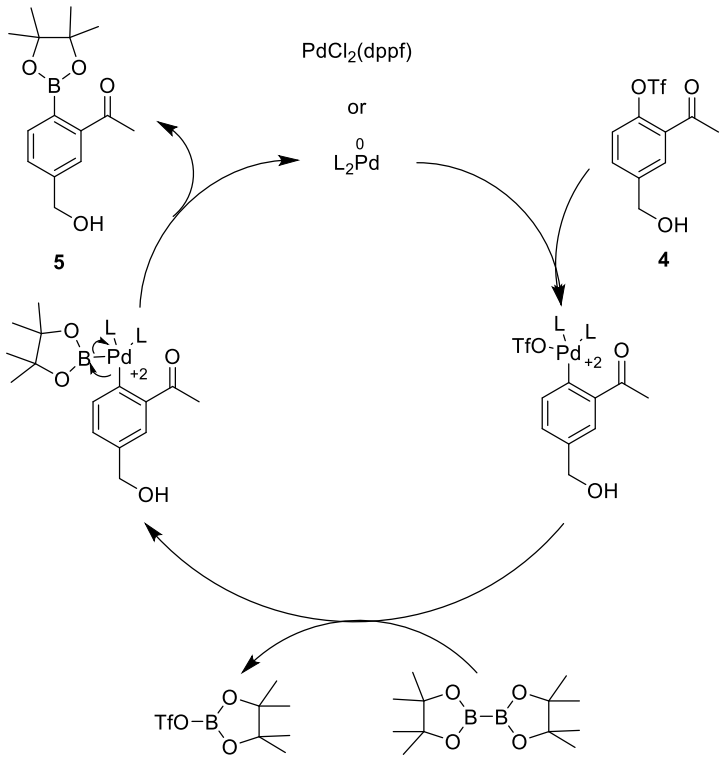


Figure 17. Compound 5 synthetic mechanism.

At this point, and according to the literature, (10) we had the most important scaffold of the final molecule, which is already able to form an iminoboronate by interacting with a lysine residue. However, we still needed to synthetise a molecule that could react with two equivalents of compound **5** in order to obtain the desired linker.

We defined from the beginning that we were going to use a diamine as the linker between both compounds **5**, more specifically the N^1,N^2-dimethylethane-1,2-diamine. Nevertheless, the direct reaction between a hydroxyl group and an amine cannot occur so we needed to find out a way to get around this problem. We then decided to react our diamine with 4-nitrophenyl chloroformate to obtain compound **7**. Compound's **7** features two *para*-nitrophenylcarbamate groups on both ends of the molecule, that allow the attack of compound's **5** hydroxymethyl group on the carbonyl group of the carbamate, leading to the release of *para*-nitrophenol and the formation of a stabler carbamate. In the end, after purification of the reaction crude, we obtained a mixture of compound **7** and other impurities. Because we were not able to obtain compound **7** in its pure form, it was not possible to perform the final reaction to yield compound **8**.

Despite the failing to synthetize compound **8**, we were able to successfully obtain one of the precursors (compound **5**) and prove that, with some adjustments to the reactional

conditions, we could also obtain the second one (compound **7**), which gives us hope that with a few small reactional adjustments in the synthetic process, compound **8** can be obtained. However, it is also possible to insert a fluorescent probe or a cytotoxic drug in the same position of the hydroxymethyl group of compound **5**, which we can try to synthetize in a future work, since we already have compound **5**.

3.2 Diazaborines and ADCs

We were also inspired by the previous studies on DABs as part of the bioconjugate engineering process, to develop the next approach of our work. (32,33) Containing a boronic acids *ortho* to a carbonyl, and this group a precursor to the formation of DABs, we also decided to prove that compound **5**, apart from the ability to act as a precursor to form an iminoboronate, could also be a part of an ADC linker's scaffold.

Theoretically, the hydroxymethyl group of compound **5** would be able to be modified with a scaffold containing a maleimide at the end, which can interact with the antibody, while the boronic acid *ortho* to the carbonyl would be able to react with a hydrazine containing a payload (fluorescent probe or cytotoxic drug), forming a diazaborine, and subsequently leading to the formation of an ADC linker (Figure 18). However, we needed to prove that the formation of diazaborines was possible using different boronic acids scaffolds, as well as hydrazines containing different substituents, and if the yields of these reactions were acceptable for what we were trying to achieve.

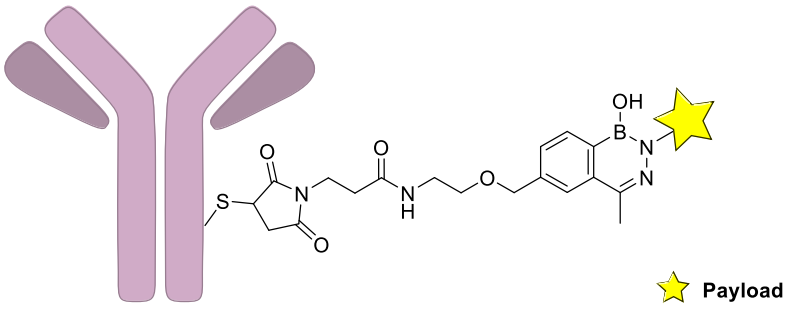
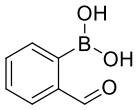
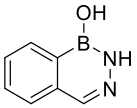
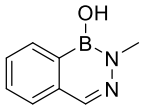
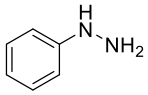
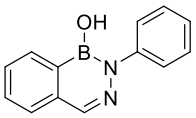
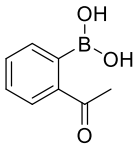
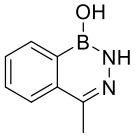
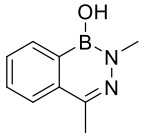
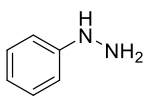
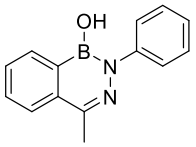


Figura 18. Example of an antibody-drug conjugate containing compound 5 as part of the linker

We then performed a set of testing reactions using two boronic acids scaffolds (2-FBBA and 2-ABBA) and three hydrazines (hydrazine hydrate, methylhydrazine and phenylhydrazine) as reactants. We reacted each BA with each hydrazine, using water

or methanol as solvents, at room temperature, for up to 2h. In the end we were able to isolate all the products of the six different reactions with moderate to good yields (Table 1).

Table 1. Products and yields of the different reactions.

Reactants		**products**	**Yields**
BAs	**Hydrazines**	**DABs**	
 2-FBBA	 Hydrazine hydrate	 Compound **9**	83%
	 Methylhydrazine	 Compound **10**	67%
	 Phenylhydrazine	 Compound **11**	49%
 2-ABBA	 Hydrazine hydrate	 Compound **12**	42%
	 Methylhydrazine	 Compound **13**	29%
	 Phenylhydrazine	 Compound **14**	35%

Despite the fact that the formation of the diazaborine is faster and more efficient using boronic acids ortho to a formyl group than ortho to an acetyl group, as well as using hydrazines with smaller substituent groups, we were still encouraged to perform the reaction between our previously synthetized compound **5** and a hydrazine to find out if the formation of a diazaborine could also occur, proving if we could use compound **5** also as part of an ADC linker.

To prove this, we reacted compound **5** with one of the hydrazines that we previously used (phenylhydrazine), on a small scale. The reaction proceeded in an Eppendorf tube for 10 min and analysed by low resolution mass spectrometry (LRMS). We found out that the reaction was possible and that we had obtained compound **15** (Figure 19), meaning that our compound **5**, when reacted with hydrazines yields a DAB, and can be used as part of an ADC linker.

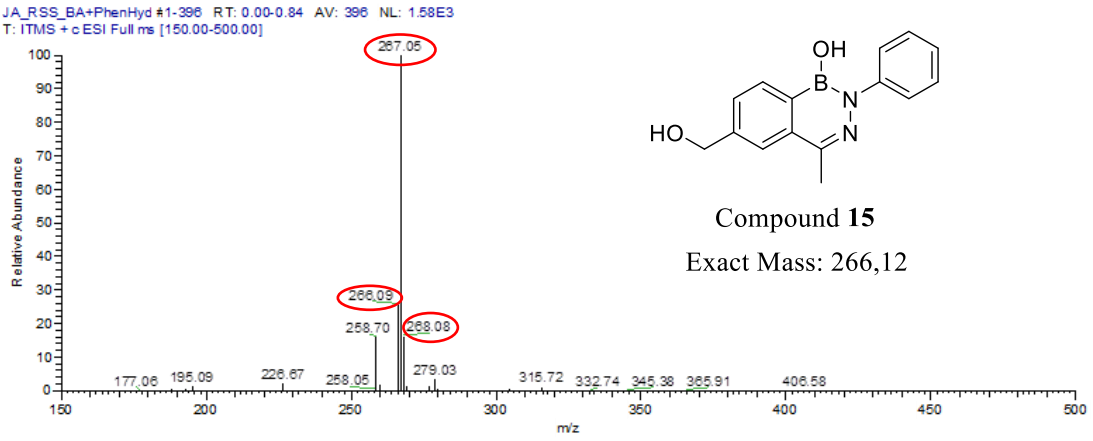


Figura 19. Structure, molecular weight and LRMS obtained peaks of compound 15.

4 Conclusions

In this project we were successfully able to synthetise a bivalent molecule (compound **5**) that can be used in two different therapeutics approaches to cure cancer, as part of an inter-protein linker that can be used to form a protein aggregate that incapsulates a cytotoxic drug, or as part of an ADC linker that carries the cytotoxic drug to a specific tissue. The bivalence of this molecule is allowed because of the boronic acid *ortho* to a carbonyl group that is present in its scaffold. This specific group is able to form a stable iminoboronate with a *N*-terminal lysine residue of a protein, as well as diazaborine when reacted with a hydrazine.

In a future work we want synthetise the final inter-protein linker, by reacting two equivalents of our molecule with a diamine linker, and to obtain a functional ADC linker, by reacting our molecule with a hydrazine containing a prodrug to form a diazaborine, as well as, with a molecule containing maleimide in the end of its scaffold. Afterwards, we want to perform biological assays to study the behaviours of these molecules in physiological conditions.

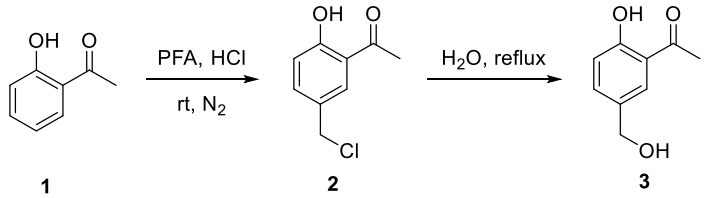
5 Materials and Methods

All reactions were performed in oven-dried glassware. All the water used as solvent in the reactions was previously distilled. The reactants were purchased from commercial sources and used without further purification.

Reaction mixtures were analysed by thin layer chromatography TLC using coated silica gel plates (Merck, aluminium sheets, silica gel 60 F254), and visualization of TLC spots was performed using UV-light. NMR spectra were recorded in a Bruker AMX 300 using $CDCl_3$ and DMSO as deuterated solvents. All coupling constants (*J* values) are expressed in Hz and chemical shifts (δ) in ppm.

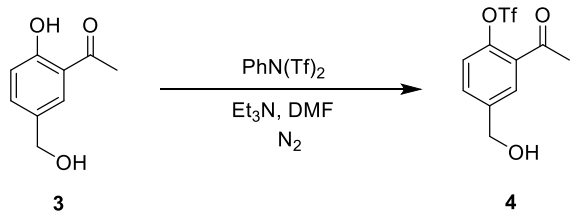
Low resolution mass spectra were recorded on a LCQ Fleet Ion Trap Mass Spectrometer (Thermo Fisher Scientific, Germany), equipped with a triple quadrupole and an electrospray ion source (ESI) operating in positive mode using Milli-Q water as solvent.

5.1 Synthesis of 1-(2-hydroxy-5-(hydroxymethyl)phenyl)ethan-1-one (3)



1-(2-hydroxyphenyl)ethan-1-one (9.87 mL, 81.17 mmol) was slowly added portion wise to a solution of paraformaldehyde (4.87 g, 162.34 mmol) in HCl 37% (50 mL, 2.25 mol). The mixture was stirred at room temperature under nitrogen atmosphere for 48 h. The resulting precipitate was filtered, washed with NaOH 1 M, and dried under vacuum. The yellow solid was stirred in 250 mL of refluxing water for 12h, after which the solvent was concentrated *in vacuo* and the crude purified by column chromatography [hexane:EtOAc (6:4-7:3)] to give **3** as a light yellow solid (6,523 mg, 39.25 mmol, 48%). **^{1}H NMR:** (300 MHz, Chloroform-*d*) δ 12.17 (d, *J* = 0.4 Hz, 1H, OH), 7.71 – 7.61 (m, 1H, CH_{arom}), 7.40 (ddd, *J* = 8.5, 2.2, 0.5 Hz, 1H, CH_{arom}), 6.91 (d, *J* = 8.5 Hz, 1H, CH_{arom}), 4.59 (s, 2H, CH_2), 2.58 (s, 3H, CH_3).

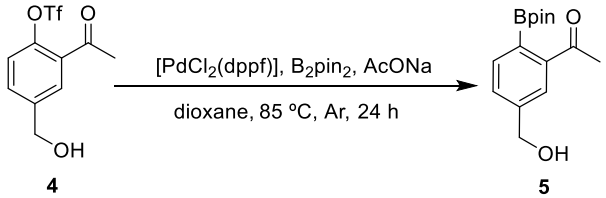
5.2 Synthesis of 2-acetyl-4-(hydroxymethyl)phenyl trifluoromethanesulfunate (4)



3 4

A mixture of 1-(2-hydroxy-5-(hydroxymethyl)phenyl)ethan-1-one (1.133 g, 6.70 mmol), N-phenyl bistrifluoromethane sulfonimide (2.87 g, 8.04 mmol) and dry triethylamine (1.86 mL, 13.40 mmol) in dimethylformamide (5 mL) was stirred under nitrogen atmosphere. The reaction mixture was stirred for 72 hours, after which the solvent was concentrated *in vacuo* and the crude purified by column chromatography [hexane:EtOAc (6:4)] to give **4** as a light orange oil(986 mg, 3.31 mmol, 49%). **^{1}H NMR**: (300 MHz, Chloroform-*d*) δ 7.77 – 7.71 (m, 1H, CH_{arom}), 7.70 – 7.66 (m, 0.1H), 7.51 (dd, *J* = 8.5, 2.3, 0.8 Hz, 1H, CH_{arom}), 7.40 (dd, *J* = 8.6, 2.2 Hz, 0.1H), 7.26 – 7.22 (d, 1H, CH_{arom}), 6.89 (d, *J* = 8.5 Hz, 0.1H), 4.71 (s, 2H, CH_2), 4.58 (s, 0.2H), 2.56 (s, 3H, CH_3), 1.97 (s, 0.9H).

5.3 Synthesis of 1-(5-(hydroxymethyl)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)ethan-1-one (5)

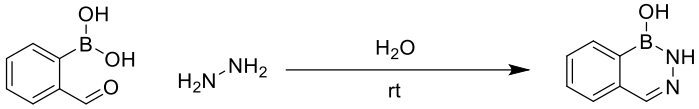


4 5

According to the procedure reported in the literature, (12) a mixture of 2 (500 mg, 1.68 mmol), bis(pinacolato)diboron (851 mg, 3.35 mmol), [1,1'-bis(diphenylphosphino)ferrocene]palladium (II) chloride (124 mg, 0.17 mmol), and sodium acetate (419 mg, 5.04 mmol) were added to dioxane (15 mL) in a previously flame dryed flask. The dioxane was degassed with bubbling argon. The reaction was

stirred for 12 hours under 90 oC under argon atmosphere. The solvent was then evaporated *in vacuo* and the crude was purified by column chromatography [CH_2Cl_2:EtOAc (9:1 – 8:2)] to give 3 as an orange oil (293 mg, 1.06 mmol, 63%). **^{1}H NMR:** (300 MHz, Chloroform-*d*) δ 7.79 – 7.69 (m, 1H, CH_{arom}), 7.41 (m, *J* = 1.3 Hz, 2H, CH_{arom}), 4.68 (s, 2H, -CH_2OH), 2.53 (d, *J* = 1.8 Hz, 3H, -COCH3), 1.37 (q, *J* = 1.3, 0.7 Hz, 12H, CH_3). **^{13}C NMR**: (75 MHz, Chloroform-*d*) δ 199.9, 142.1, 140.9, 132.2, 130.4, 126.4, 83.7, 83.0, 75.0, 64.3, 60.4. **LR ESI$^+$:** m/z $[M+H]^+$ expected: 276,1; m/z $[M+H]^+$ found: 277,0.

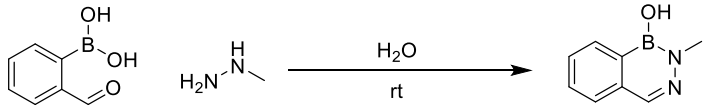
5.4 Synthesis of benzo[*d*][1,2,3]diazaborinin-1(2*H*)-ol (9)



9

A suspension of (2-formylphenyl)boronic acid (105 mg, 0.70 mmol) in distilled water (7 mL) was stirred for 10 min at room temperature until the total dissolution of the boronic acid, after which hydrazine hydrate (34.8 μL, 0.70 mmol) was added. The solution was then stirred at room temperature for another 45 minutes. The reaction was filtered, obtaining the desired compound. The compound was dried *in vacuo*, obtaining **1** as a white solid (85 mg, 0.58 mm 83%). **^{1}H NMR:** (300 MHz, DMSO-*d*6) δ 9.90 (s, 1H, OH), 8.17 (d, *J* = 8.5 Hz, 2H, CH_{arom}), 7.98 (d, *J* = 0.7 Hz, 1H, CH_{arom}), 7.74 – 7.65 (m, 2H, CH_{arom}), 7.57 (ddd, *J* = 7.5, 5.4, 3.0 Hz, 1H, CH_{arom}). **LR ESI$^+$:** m/z $[M+H]^+$ expected: 146,0; m/z $[M+H]^+$ found: 147,0.

5.5 Synthesis of 2-methylbenzo[*d*][1,2,3]diazaborinin-1(2*H*)-ol (10)

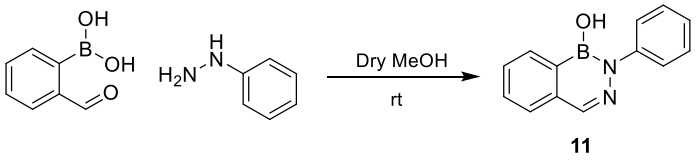


10

A suspension of (2-formylphenyl)boronic acid (115 mg, 0.77 mmol) in distilled water (8 mL) at room temperature was stirred for 10 min until the boronic acid was completely dissolved. Once dissolved, methylhydrazine (41.2 μL, 0.77 mmol) was added to the mixture. The solution was stirred for 1 hour at room temperature. The mixture was then filtered, obtaining a precipitate. The solid was dried *in vacuo*, obtaining **10** as a white

precipitate (82 mg, 0.51 mmol, 67%). **^{1}H NMR**: (300 MHz, DMSO-*d*6) δ 8.52 (s, 1H, OH), 8.24 (ddt, *J* = 7.6, 1.5, 0.8 Hz, 1H, CH_{arom}), 7.98 (d, *J* = 0.8 Hz, 1H, CH_{arom}), 7.76 – 7.62 (m, 2H, CH_{arom}), 7.57 (ddd, *J* = 7.5, 6.6, 1.8 Hz, 1H, CH_{arom}), 3.51 (s, 3H, CH_3); **LR ESI$^+$:** m/z $[M+H]^+$ expected: 160,0; m/z $[M+H]^+$ found:161,0.

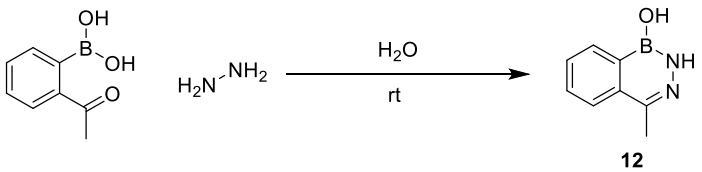
5.6 Synthesis of 2-phenylbenzo[*d*][1,2,3]diazaborinin-1(2*H*)-ol (11)



11

(2-Formylphenyl)boronic acid (90 mg, 0.60 mmol) and phenylhydrazine (61.2 µL, 0.60 mmol) were added to dry methanol (25 mL) while stirring at room temperature. The solution was stirred for 50 min at room temperature. The solvent was then removed by evaporation, and the residue was purified by column chromatography on silica gel eluting with DCM and EtOAc (6:4), obtaining **11** as a dark yellow solid (65 mg, 0.29 mmol, 49%). **^{1}H NMR**: (300 MHz, DMSO-*d*6) δ 8.94 (s, 1H, OH), 8.39 (d, *J* = 7.7 Hz, 1H, CH_{arom}), 8.20 (d, *J* = 0.7 Hz, 1H, CH_{arom}), 7.84 – 7.73 (m, 2H, CH_{arom}), 7.66 (td, *J* = 7.2, 1.7 Hz, 1H, CH_{arom}), 7.59 – 7.54 (m, 2H, CH_{arom}), 7.45 – 7.36 (m, 2H, CH_{arom}), 7.25 – 7.17 (m, 1H, CH_{arom}). **LR ESI$^+$:** m/z $[M+H]^+$ expected: 222,0; m/z $[M+H]^+$ found: 223,0.

5.7 Synthesis of 4-methylbenzo[*d*][1,2,3]diazaborinin-1(2*H*)-ol (12)

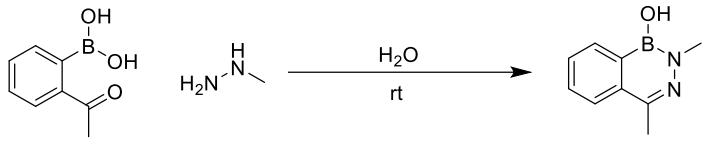


12

A suspension of (2-acetylphenyl)boronic acid (82 mg, 0.48 mmol) in distilled water (5 mL) was stirred for 15 min at room temperature until total dissolution, after which the hydrazine hydrate (24.1 µL, 0.48 mmol) was added. The mixture was then stirred at

room temperature for 1 hour and 10 minutes. The reaction was filtered and washed, obtaining a precipitate. The compound was then dried *in vacuo*, obtaining **12** as a white compound (32 mg, 0.20 mmol, 42%). **^{1}H NMR:** (300 MHz, DMSO-*d*6) δ 9.62 (s, 1H, OH), 8.18 (ddd, *J* = 7.5, 1.5, 0.7 Hz, 1H, CH_{arom}), 8.01 (s, 1H, CH_{arom}), 7.84 – 7.67 (m, 2H, CH_{arom}), 7.58 (td, *J* = 7.3, 1.3 Hz, 1H, CH_{arom}), 2.44 (s, 3H, CH_3). **LR ESI$^+$:** m/z $[M+H]^+$ expected: 160,0 ; m/z $[M+H]^+$ found: 161,0

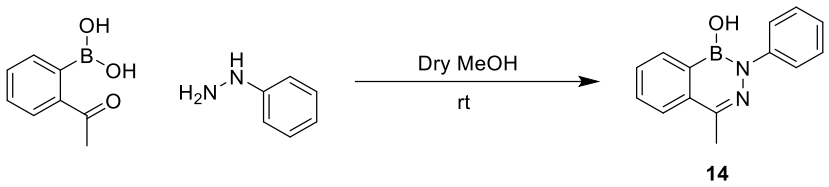
5.8 Synthesis of 2,4-dimethylbenzo[*d*][1,2,3]diazaborinin-1(2*H*)-ol (13)



13

(2-Acetylphenyl)boronic acid (84 mg, 0.50 mmol) was stirred in distilled water (5 mL) at room temperature for 15 min. After the dissolution of the boronic acid, methylhydrazine (26.8 µL, 0.50 mmol) was added. The solution stirred for 1 hour and 10 minutes at room temperature. The mixture was filtered, obtaining the desired compound. The product was dried *in vacuo*, obtaining **13** as a white solid (24 mg, 0.14 mmol, 27%). **^{1}H NMR:** (300 MHz, DMSO-d6) δ 8.34 (s, 1H, OH), 8.25 (ddd, *J* = 7.5, 1.5, 0.7 Hz, 1H, CH_{arom}, 7.80 (dt, *J* = 8.0, 1.0 Hz, 1H, CH_{arom}), 7.75 – 7.66 (m, 1H, CH_{arom}), 7.58 (td, *J* = 7.3, 1.2 Hz, 1H, CH_{arom}), 3.45 (s, 3H, CH_3), 2.45 (s, 3H, CH_3). **LR ESI$^+$:** m/z [M+H]+ expected: 174.0 ; m/z $[M+H]^+$ found: 175,0.

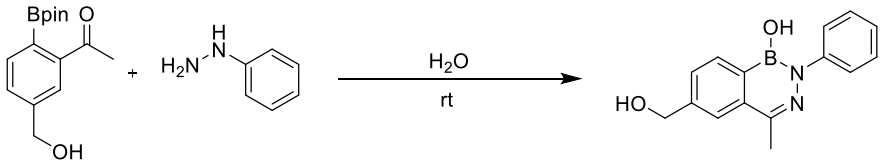
5.9 Synthesis of 4-methyl-2-phenylbenzo[d][1,2,3]diazaborinin-1(2*H*)-ol (14)



14

Under stirring at room temperature, (2-acetylphenyl)boronic acid (49 mg, 0.28 mmol) and phenylhydrazine (29.0 μL, 0.28 mmol) were added to dry methanol (6 mL). The mixture was then stirred for 45 min at room temperature. The reaction was then purified by column chromatography on silica gel [CH_2Cl_2:EtOAc (6:4)] eluting with CH obtaining **14** as a dark orange solid residue (23 mg, 0.10 mmol, 35%).

5.10 Synthesis of 6-(hydroxymethyl)-4-methyl-2-phenylbenzo[*d*][1,2,3]diazaborinin-1(2*H*)-ol (15)



5 **15**

1-(5-(hydroxymethyl)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)ethan-1-one (1 mg, 3.62 μmol) was added to water (362 μL) obtaining a solution with a concentration of 10 mM. We prepared another solution with the same concentration, using phenylhydrazine (2 mg, 18.49 μmol) and water (1.85 mL). Afterwards we added 25 μL of both solutions to an Eppendorf tube and completed with water until we had 500 μL. The reaction was left for 10 min at room temperature, and then injected in the low mass spectrometer to be analysed. **LR ESI$^+$:** m/z [M+H]+ expected: 266.1; m/z $[M+H]^+$ found: 267,0.

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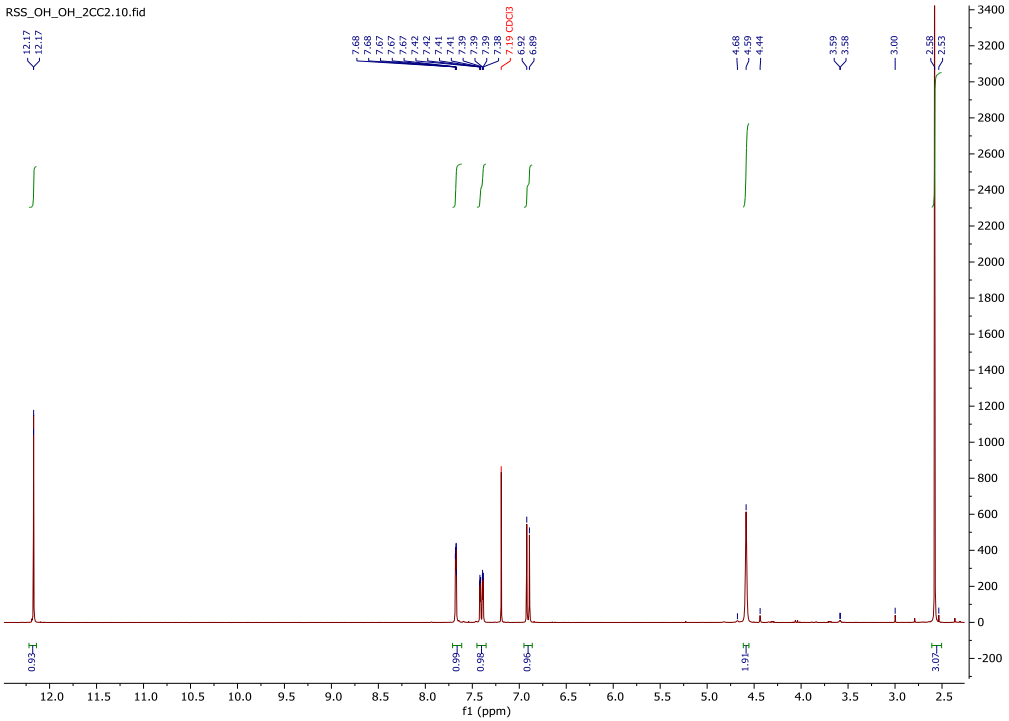
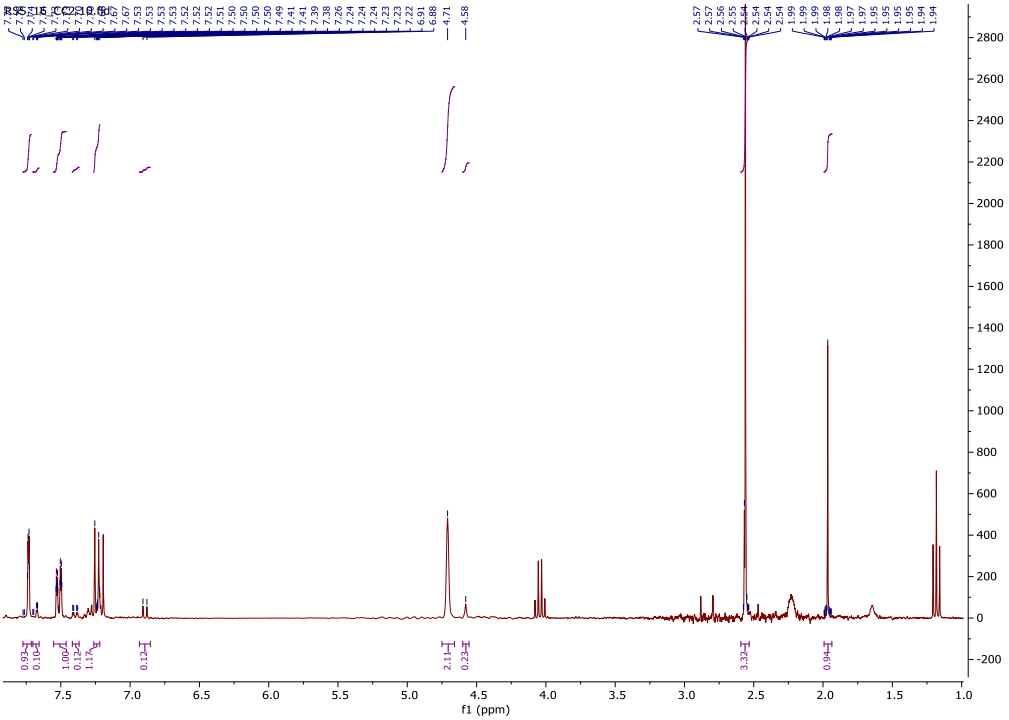
Appendix

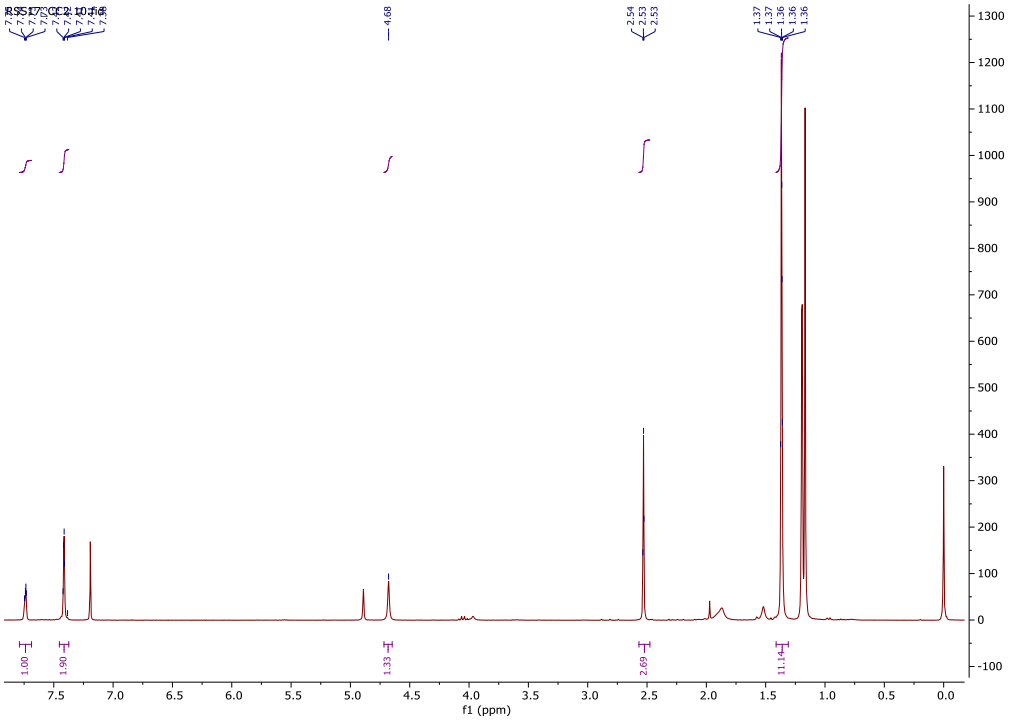
A1. ^{1}H NMR of 1-(2-hydroxy-5-(hydroxymethyl)phenyl)ethan-1 one (3)



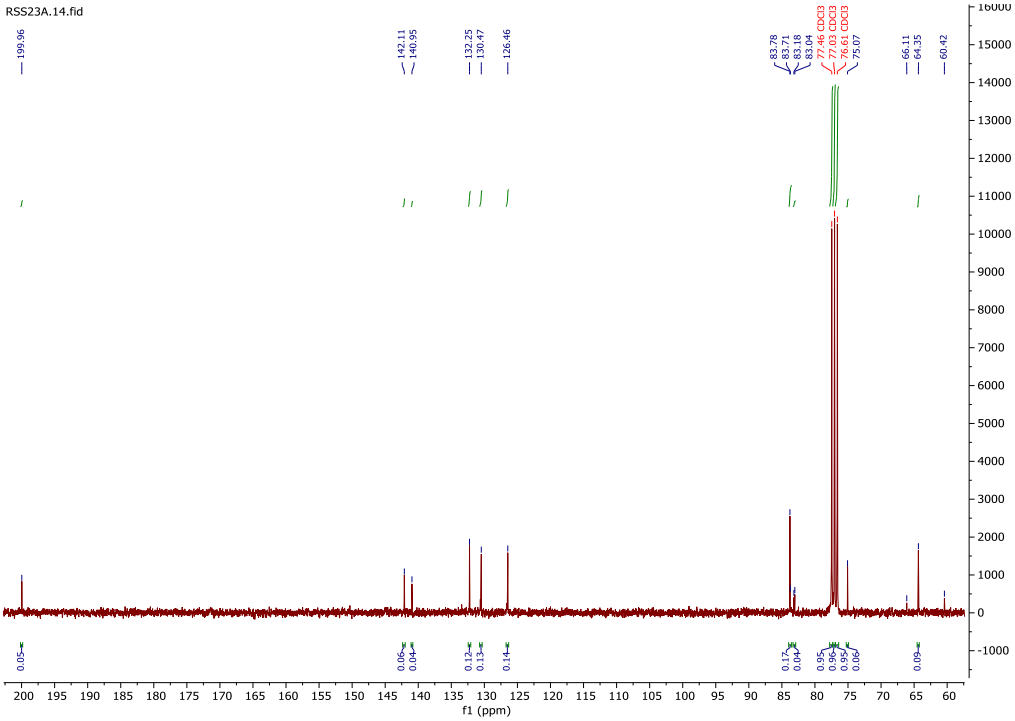
A2. ^{1}H NMR of 2-acetyl-4-(hydroxymethyl)phenyl trifluoromethanesulfunate (4)



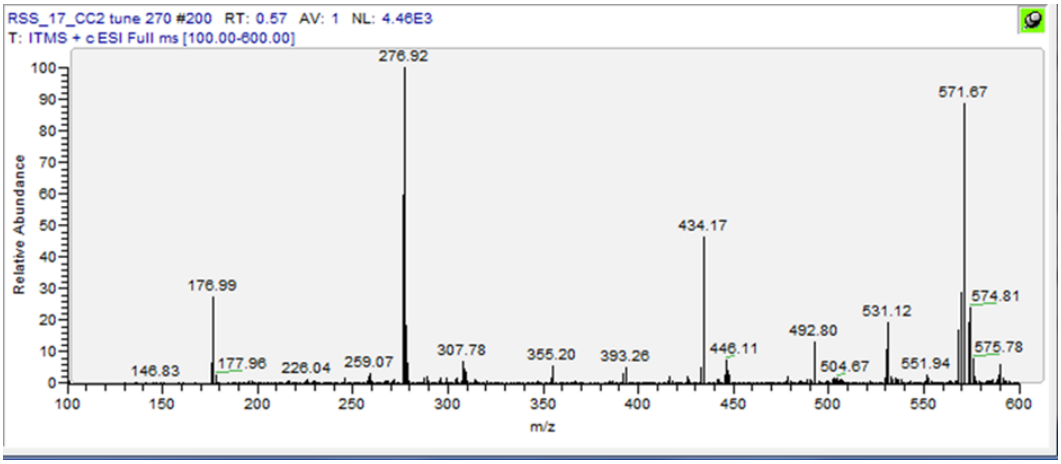
A3. ^{1}H NMR of 1-(5-(hydroxymethyl)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)ethan-1-one (5)



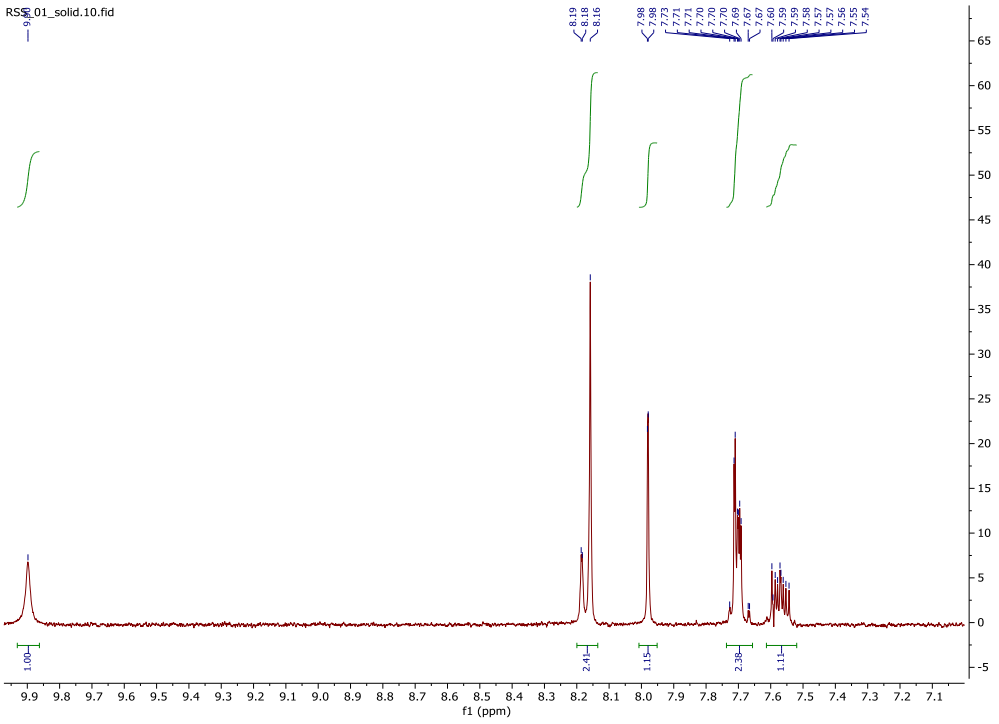
A4. ^{13}C NMR of 1-(5-(hydroxymethyl)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)ethan-1-one (5)



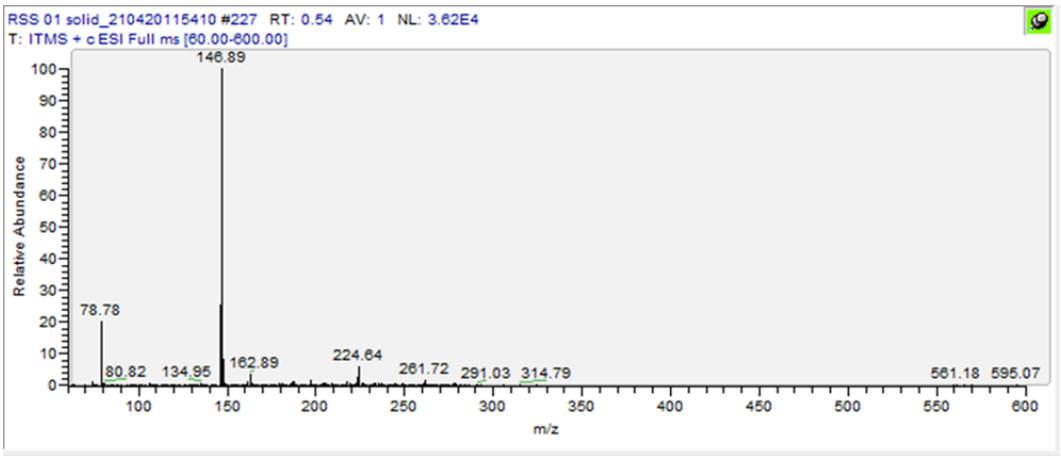
A5. LRMS of 1-(5-(hydroxymethyl)-2-(4,4,5,5-tetramethyl-1,3,2-dioxaborolan-2-yl)phenyl)ethan-1-one (5)



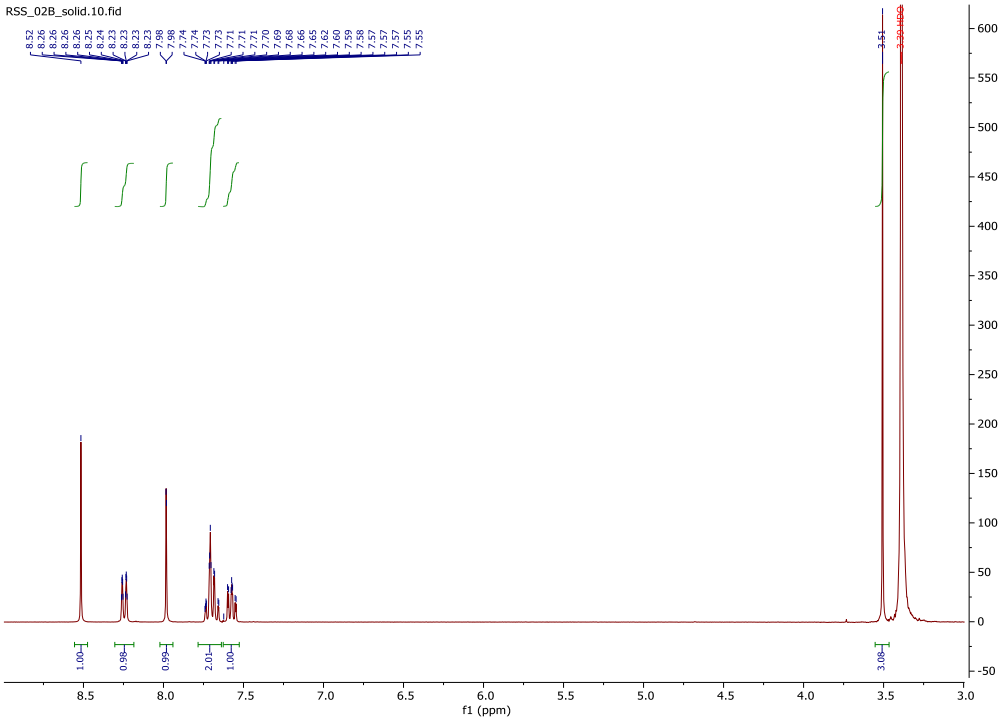
A6. ^{1}H NMR of benzo[*d*][1,2,3]diazaborinin-1(2H)-ol (9)



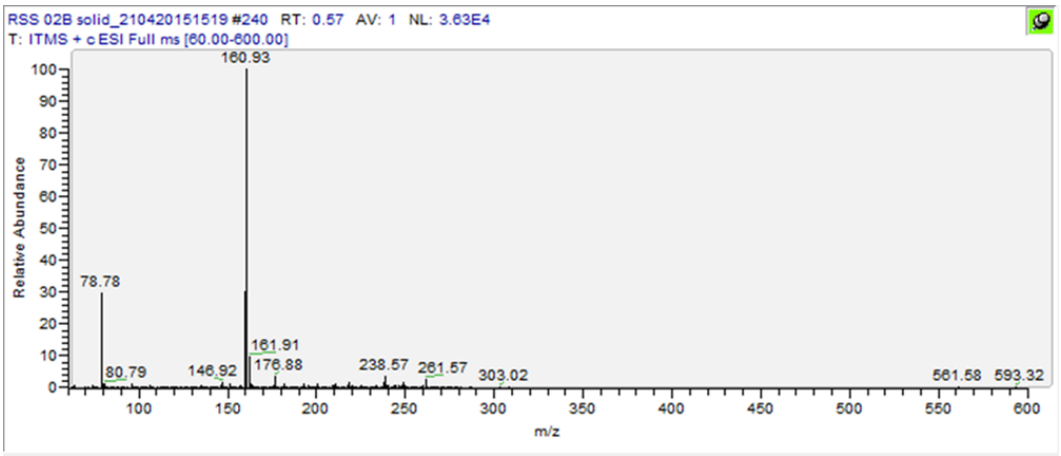
A7. LRMS of benzo[*d*][1,2,3]diazaborinin-1(2H)-ol (9)



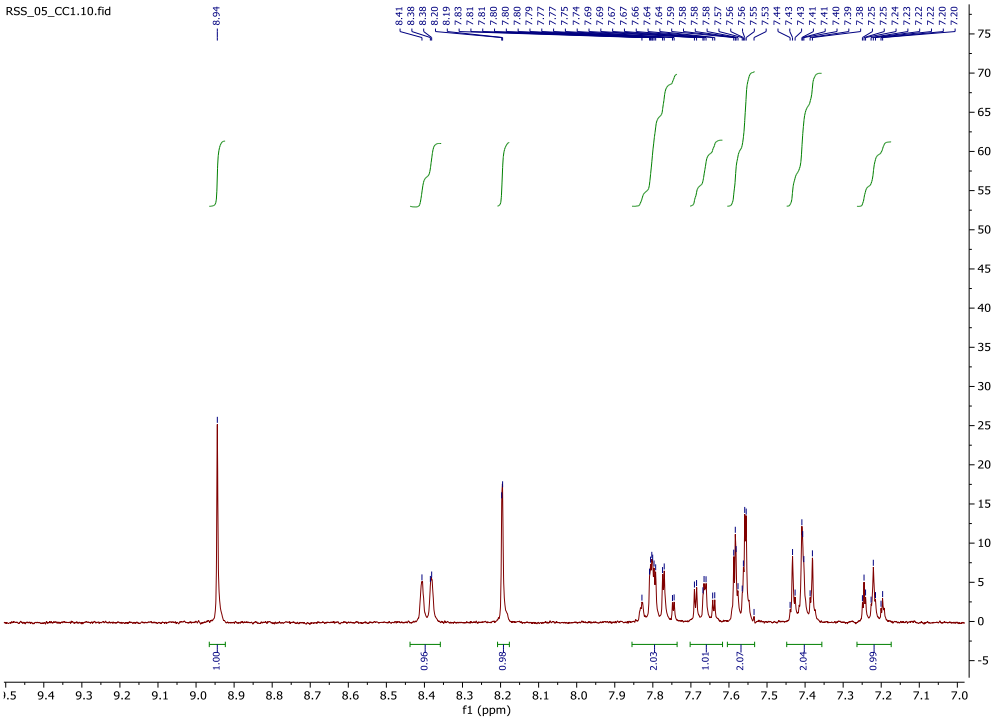
A8. ^{1}H NMR of 2-methylbenzo[*d*][1,2,3]diazaborinin-1(2H)-ol (10)



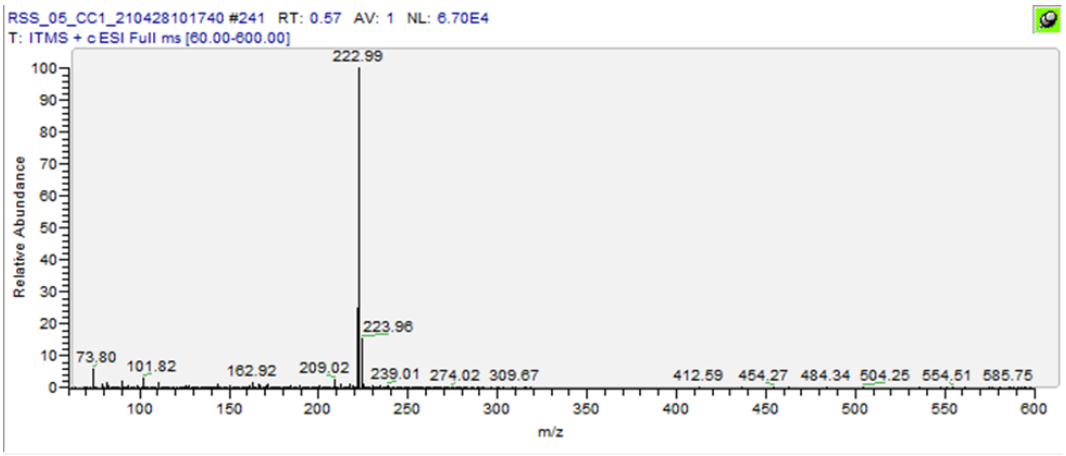
A9. LRMS of 2-methylbenzo[*d*][1,2,3]diazaborinin-1(2H)-ol (10)



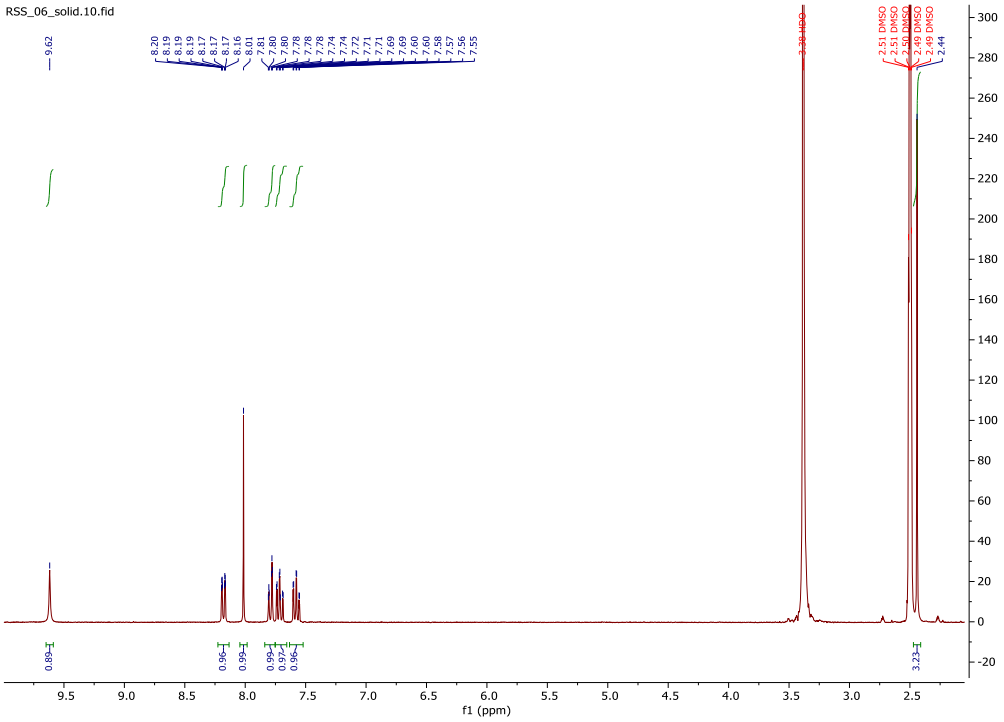
A10. ^{1}H NMR of 2-phenylbenzo[*d*][1,2,3]diazaborinin-1(2H)-ol (11)



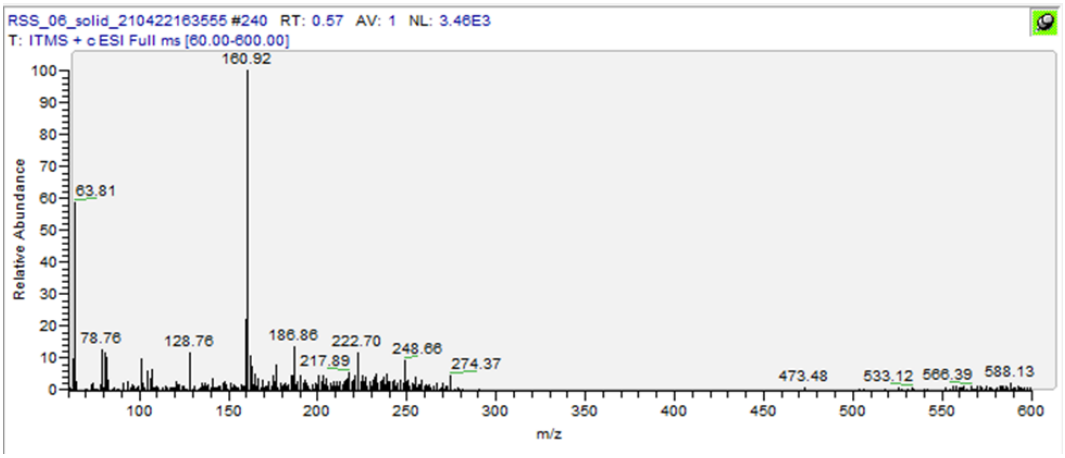
A11. LRMS of 2-phenylbenzo[*d*][1,2,3]diazaborinin-1(2H)-ol (11)



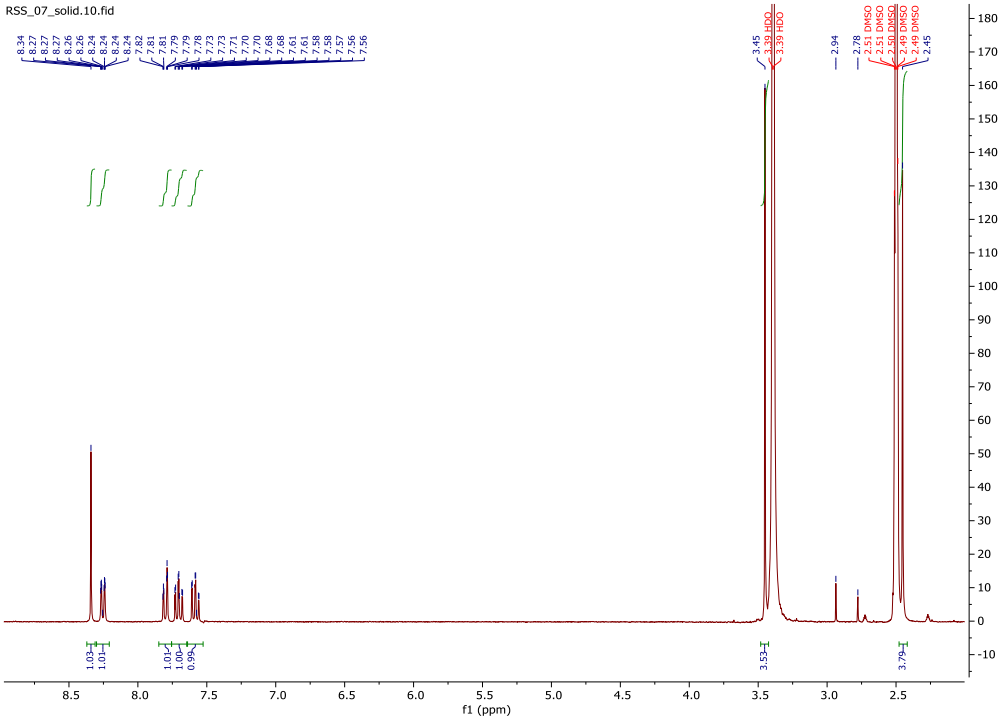
A12. ^{1}H NMR of 4-methylbenzo[*d*][1,2,3]diazaborinin-1(2H)-ol (12)



A13. LRMS of 4-methylbenzo[*d*][1,2,3]diazaborinin-1(2H)-ol (12)



A14. ^{1}H NMR of 2,4-dimethylbenzo[*d*][1,2,3]diazaborinin-1(2H)-ol (13)



A15. LRMS of 2,4-dimethylbenzo[*d*][1,2,3]diazaborinin-1(2H)-ol (13)