Published in final edited form as:

Org Biomol Chem. 2017 September 20; 15(36): 7543–7548. doi:10.1039/c7ob01708a.

Carbohydrazides and *ortho*-formylphenylboronic acid: Formation of hydrazones and stabilized boron-nitrogen heterocycles in aqueous solution

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Abstract

A recent addition to the suite of fast bioorthogonal reactions combines hydrazine and hydroxylamines with *ortho*-carbonyl substituted phenylboronic acids. Carbohydrazides are easily incorporated into biomolecules and are therefore may be attractive partners in these reaction. Here we show that simple alkyl carbohydrazides form a single product with *ortho*-formylphenylboronic acid in organic solvent and in solid state. The solution structures of the products formed from the carbohydrazides in buffered aqueous solution, however, are markedly different than those identified in organic solvent and solid state. The reactants form a mixture of hydrazone and heterocyclic products, the relative composition of which varies with pH. The observed reversibility of bioconjugates using carbohydrazide can thus be explained by the reversibility of the boron-nitrogen bond in the heterocycle. In contrast, inclusion of an α -amine to the carbohydrazide substrate yields a single product in which both nitrogens are bonded to boron. These tricyclic structures are the same in organic solvent, solid state and aqueous solution from pH 4 to pH 9. Bioconjugates formed with α -amino carbohydrazides are stable to SDS-PAGE, while those formed with simple alkyl carbohydrazides are not. We propose that inclusion of an intramolecular stabilizing ligand in a carbohydride substrate is a generally applicable principle that may be exploited to form boronic acid-based bioconjugates with defined structure and resistance to hydrolysis.

Introduction

Reactions of hydrazines and hydroxylamines with *ortho*-carbonyl substituted phenylboronic acids are of recent interest in bioorthogonal chemistry. We and others have shown that the boronic acid moiety accelerates the rate of product formation by about three orders of magnitude over that of the unsubstituted aldehyde.^{1–4} When the nucleophile is a hydrazine,

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the product formed was presumed to be a hydrazone.² We were the first to discover that the final product may also be a substituted 1,2-dihydro-1-hydroxy-2,3,1-benzodiazaborine (DAB; Scheme 1), an unusual heterocycle produced by dehydration of the hydrazone. The DAB is in fact the only product formed when an aromatic hydrazine undergoes reaction with 2-formylphenylboronic acid (**1**), even in very dilute (low micromolar) aqueous solution.⁴ Subsequently, similar structures were shown to be formed in dilute aqueous solution with other aromatic hydrazines³ and, more recently, with semicarbazides.⁵

The DABs form stable bioconjugates: for example, protein conjugates via a DAB linkage are stable to SDS-PAGE.⁴ In contrast, some products formed between hydroxylamines and hydrazines and 2-formyl- or 2-acetylphenylboronic acids have been reported to be reversible at neutral pH.^{1, 2} Rational design of bioorthogonal coupling reagents for specific applications is bolstered by understanding the fundamental chemistry of the reactions under biologically relevant conditions.⁶ It is important to understand the outcome of the reaction and the structure(s) of the product(s) under conditions relevant to a biological system. Since carbohydrazides can easily be incorporated into proteins^{7, 8} and nucleic acids,⁹ characterization of reactions with this functional group is significant to many potential bioconjugation applications.

Until now, detailed study of reactions between carbohydrazides and 2-formylphenylboronic acid (**1a**) or 2-acetylphenylboronic acid (**1b**) have been performed in organic solvent.¹⁰ In this work, we evaluate reactions and products of **1a** with model hydrazides **2a-d** in neutral aqueous solution. We find that simple carbohydrazides form a boron-nitrogen heterocycle when precipitated from aqueous solution, but the bond between the boron and nitrogen in the product is reversible in a pH-dependent manner in aqueous solution. In contrast, α -amino carbohydrazides form tricyclic products containing two boron-nitrogen bonds and are stable in aqueous solution at both acidic and basic pH. Such ligands can be used to form highly stable bioconjugates.

Results and discussion

Products with alkyl hydrazides **2a** and **2b**

Kanichar et al.¹¹ have condensed *ortho*-carbonyl substituted phenylboronic acids with various hydrazide-containing substances and studied the structure of the product(s) by NMR and X-ray crystallography. They observed that the structure of the product was sensitive to reaction conditions (aqueous ethanol at room temperature or reflux in acetonitrile) and, in some cases, the nature of the solvent used for NMR spectroscopy. Of the 23 carbohydrazides tested, only 20% yielded a hydrazone (**3**) as the final product. The remaining molecules yielded a few 2-acylated-1,2-dihydro-1-hydroxy-2,3,1-benzodiazaborines (**6**), but most products were anhydro dimers of the DABs (**5**) (Scheme 2).

To assess the reactivity of **1a** with **2a** in aqueous solution, the reaction was first performed using high concentrations of each component (125 mM) in pH 7.4 sodium phosphate buffer. A white precipitate appears upon mixing the two solutions. The resulting solid was dried and isolated without the use of heat. Even under these mild conditions, the product of the reaction was the anhydro dimer (**5a**, Scheme 2). ¹H and ¹³C NMR spectra are in good

agreement with published data for **5a**.¹¹ The ¹¹B NMR spectrum, not previously reported, displays a single peak at δ 0.62, consistent with tetracoordinate boron bonded to good δ -donating ligands.¹²

A second simple hydrazide, propanoic acid hydrazide (**2b**), was allowed to react with **1a** under similar aqueous conditions. Once again the product was an anhydro dimer (**5b**). Notable in the ¹H NMR spectrum of **5b** is the coupling pattern of the methylene protons of the propyl substituent (d of q at δ 2.96 and 2.79, Figure 1), indicating protons in a prochiral environment. Addition of a drop of D₂O to the sample yielded a second set of resonances for the methylene protons (q at δ 3.10), expected for monomeric form of the DAB (**6b**). The ¹¹B NMR spectra support the conclusions drawn from the ¹H NMR spectra (Figure 1, right panels). A peak at δ 1.0 is consistent with the anhydro dimer DAB (**5b**), in which the boron atom is tetracoordinate. The chemical shift of the downfield peak (δ 28) is in a spectral region associated with sp²-hybridized boronic acids and DABs. ($\sim\delta$ 26 – 30).¹³

Solutions of **5a** and **5b** were allowed to slowly crystallize at room temperature. X-ray analysis confirmed the structures deduced from NMR spectra (Figure 2). Both molecules display axial chirality in the crystal, which is anticipated.¹¹ The asymmetric cell is a racemic mixture of the two enantiomers. The boron atom is tetracoordinate, and the B-O-B bonds are maintained in solution in solvents such as deuterated DMSO, chloroform and benzene. (Spectra are shown in ESI.)

We therefore conclude that the precipitates formed from the reactions of **1a** with **2a** or **2b** in concentrated aqueous solution at pH 7 are 2,3,1-benzodiazaborines that have undergone dehydration to form anhydro dimers (**5a** or **5b**). Dimers may hydrolyze to a monomeric DAB (**6a** or **6b**) in organic solvent containing a small amount of water, but a hydrazone product (**3**) was not observed under the conditions tested. Apparently the intramolecular B-O bonding with the carbohydrazide carbonyl oxygen stabilizes the product in the solid state, regardless of whether it is prepared from aqueous solution or organic solvent.

The next task was to assess the reactions of **1a** with **2a** or **2b** in environments relevant to biological applications, dilute aqueous solution. Equimolar solutions of **1a** and **2a** or **2b** were mixed in pH 7 buffer to a final concentration of 2 mM and NMR spectra were collected of the resulting solutions. Noting the presence of minor products in ¹H spectrum (*vide infra*), additional samples were prepared over a pH range of 4–9. Figure 3 shows regions of the ¹H NMR spectra from the product of **1a** with **2b**. Structures of the species are shown in Scheme 3.

At high pH (pH 9), a single species is observed by NMR. The structure of this product is proposed to be the hydroxylated form of the monomeric DAB (**4b**). The methylene protons are uncoupled (achiral), indicating that O-B-O bond is absent under these solution conditions (shown in spectra in ESI). The proton on the imino carbon is near δ 7.5, which is considerably upfield from the same proton of an iminoboronate formed with an aliphatic amine ($\sim\delta$ 8.5¹⁴). The chemical shift of boron is δ 1.1, which is in the same region of the ¹¹B spectrum as tetrahedral boron atom in **5b**.

At lower pH, two new species are observed. The new peaks are attributed to hydrolysis of **4b** to its corresponding hydrazone (**3b**). There are two conformational isomers of the hydrazone. [Conformational isomers are also ascribed to the product of acetylhydrazide with 2AcPBA in pH 7.4 solution.²] The imine protons of the hydrazones are δ 0.6 – 0.9 downfield from the DAB proton. These are tentatively assigned as the *E*- and *Z*-isomers, respectively, of the amide bond of the *E*-hydrazone.¹⁵ At pH 4, the lowest pH tested, the hydrazone forms are predominant. These are in equilibrium with a small amount of **1a** and **2b**. Interestingly, all species in solution are stable at the varying pHs. The proton NMR spectra of the samples remain unchanged after weeks at room temperature.

We therefore conclude that **2a** or **2b** undergo reaction with **1a** in neutral aqueous solution to form three different products, the ratios of which vary with the pH of the solution. At low pH, the hydrazone forms are the predominant product. Raising the pH shifts the equilibrium in favor of **4** (illustrated for **4b**), a hydrozylated form of a DAB. We hypothesize that Lewis acidity of the boronic acid or the Bronsted acidity of the amide-like nitrogen in **3** triggers the ring closure, with a pKa of $\sim$ 6.3. It is noted that the pKa of the corresponding nitrogen of the trifluoroacetylhydrazone of pivalaldehyde is 7.9 owing to the influence of the trifluoroacetyl group.¹⁶ It is possible that the adjacent boronic acid serves to lower the pKa of the hydrazone through non-covalent interactions.

Products with α -amino hydrazides **2c** and **2d**

The presence of the tetracoordinate boron atom in the DAB product prompted us to explore whether a coordinating ligand from an intramolecular source might promote retention of a tetracoordinate boron structure in aqueous solution. Many amino acid hydrazides are commercially available or are easily prepared from an amino acid ester. Two simple amino acid hydrazides, **2c** and **2d**, were selected for this study. High concentration (125 mM) of the carbohydrazide was combined with an equal concentration of **1a** in water. The precipitate formed with **2c** was analyzed using NMR spectroscopy. ¹H NMR spectra of the solid obtained with **2c** in DMSO-*d*₆ display some unexpected features (Figure 4). A single -OH resonance is observed, which is shifted exceptionally far upfield relative to its typical position in a planar DAB (δ 8.5)¹³. The chemical shift is consistent with that observed for the -OH of related acylated DAB.¹⁷ Note also that each methylene proton has a distinct chemical shift and is split into multiplets (d of d and d of t). The complex splitting pattern is attributed to coupling of the diastereotopic methylene protons to each other and the adjacent amine protons. Adding a drop of D₂O to the sample eliminates the peaks assigned to the hydroxyl and amine protons and reduces the methylene splitting a set of doublets. These data, coupled with ¹H-¹⁵N HMBC spectra and HRMS, support the structure of the molecule as **7c** (Scheme 4).

NMR spectra of **7c** in aqueous solution at pH 4–9 were collected. No difference in the aromatic region of the ¹H NMR spectrum was detected over the entire pH range and up to one week at room temperature. It is noted that the signals for the diastereotopic methylene protons change from doublets at pH 7 (Figure 5A) to multiplets at the lower pH (Figure 5B). We hypothesize that rate of exchange of the amine protons is slowed sufficiently at acidic pH to allow coupling of the amine protons to the methylene protons. Heating this pH 4

NMR sample to 60°C increases the rate of proton exchange, causing the peaks to coalesce into two doublets (from the diastereotopic methylene protons coupling to each other, see ESI). Further support for this hypothesis is derived from the ^1H NMR spectrum of **7d** (the amine protons of **7c** are replaced by methyl groups), where the diastereotopic methylene protons couple only to each other and appear as doublets over the entire pH range 4–9 (shown in ESI).

X-ray quality crystals were obtained for compound **7c**. Additionally, crystals were obtained for **7e** (described in ESI). These structures confirm the presence of both an intramolecular B–N bond as well as the B–N bond in the DAB ring in these molecules. (Figure 6)

Experiments to assess the potential applicability of this chemistry to bioorthogonal labeling were performed using a model protein. Bovine serum albumin (BSA) was functionalized through reduced cysteine residues to contain either a carbohydrazide or an α -amino hydrazide functional group on the protein surface. These functionalized proteins were allowed to react with a coumarin-labeled fpBA. The resulting solutions were subjected to rapid gel filtration and analyzed by SDS-PAGE (as described in ESI). Both labeled proteins maintained the fluorophore through rapid gel filtration (shown in ESI), while only the α -amine-containing conjugate withstands SDS-PAGE conditions (Figure 7). In fact, the fluorescent label remains associated with the protein in the gel after more than a week in destaining solution.

Conclusions

Simple alkyl carbohydrazides undergo reaction with **1a** to yield different products depending on reaction conditions. When the reaction is performed in organic solvent or at high concentration in aqueous solution, the preferred form is the anhydro dimer. The monomeric form of the product may also exist in organic solvent possessing a small amount of water. In aqueous solution, however, multiple products may be formed depending on the pH of the solution. The heterocyclic DAB form is dominant at high pH (8–9). Lowering the pH opens the ring to yield two isomeric forms of the hydrazone as the major species in solution. Although the equilibria are stable over the pH range examined for a long period of time, we note that solutions examined by NMR were at low mM concentration, which is considerably higher than concentrations experienced by bioconjugates.⁶

We hypothesize that the previously reported instability of bioconjugates formed with carbohydrazides and orthocarbonyl substituted phenylboronic acids² can be attributed to this pH-dependent reversibility of a boron-nitrogen bond in the conjugate. In the heterocyclic form, the imine-like carbon is contained within an electron rich ring, decreasing its susceptibility to nucleophilic attack. Protonation of the hydrazone nitrogen eliminates the stabilizing boron-nitrogen bond, which may then allow the boronic acid to serve as an intramolecular Lewis acid catalyst for hydrolysis of the resulting hydrazone.

Electronic Supplementary Information (ESI) available: Synthetic procedures, NMR spectra, crystallographic data and protein labeling procedures. See DOI: [10.1039/x0xx00000x](https://doi.org/10.1039/x0xx00000x)

Addition of an α -amino group to the substrate provides an intramolecular coordinating ligand for the boron atom, resulting in two B-N bonds in the product. These molecules are stable for weeks in acidic or basic aqueous solution and protein conjugates withstand the harsh conditions of SDS-PAGE. Here, the α -amino group provides an intramolecular coordinating ligand, stabilizing the conjugate in a manner similar to Wulff-type phenylboronic acid.¹⁸

In conclusion, we propose that to form a stable bioconjugate between carbohydrazides and *ortho*-carbonyl substituted phenylboronic acids, an intramolecular coordinating ligand should be present on the carbohydrazone. This notion is supported by preliminary results with a model protein. In this work the coordinating ligand is an α -amine; however, preliminary experiments in our lab indicate that this one example of a broader concept in this series. Assessments of the bioconjugation applications of this system are underway.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

This work was supported by NIH Grant R15 GM-102867 and by a special grant from the Harpur College Dean's Office, Binghamton University. The Regional NMR Facility (600 MHz instrument) at Binghamton University is supported by NSF (CHE-0922815). The authors thank Dr. John Lloyd, Natural Products Chemistry Section, NIDDK, NIH, for mass spectrometry analyses, Professor Mathew Vetticatt for helpful discussions, Dr. Jürgen T. Schulte for assistance in NMR data acquisition and Mr. David Tuttle for photography.

Notes and references

- Schmidt P, Stress C and Gillingham D, Chem. Sci, 2015, 6, 3329–3333. [PubMed: 29142692]
- Bandyopadhyay A and Gao J, Chem. Eur. J, 2015, 21, 14748–14752. [PubMed: 26311464]
- Stress CJ, Schmidt PJ and Gillingham DG, Org. Biomol. Chem, 2016, 14, 5529–5533. [PubMed: 26876694]
- Dilek O, Lei Z, Mukherjee K and Bane S, Chem. Commun, 2015, 51, 16992–16995.
- Bandyopadhyay A, Cambray S and Gao J, J. Am. Chem. Soc, 2017, 139, 871–878. [PubMed: 27992180]
- Saito F, Noda H and Bode JW, ACS Chem. Biol, 2015, 10, 1026–1033. [PubMed: 25572124]
- Thom J, Anderson D, McGregor J and Cotton G, Bioconj. Chem, 2011, 22, 1017–1020.
- Li Y-M, Li Y-T, Pan M, Kong X-Q, Huang Y-C, Hong Z-Y and Liu L, Angew. Chem. Int. Ed, 2014, 53, 2198–2202.
- Raddatz S, Mueller-Ibeler J, Kluge J, Waess L, Burdinski G, Havens JR, Onofrey TJ, Wang D and Schweitzer M, Nucleic Acids Res, 2002, 30, 4793–4802. [PubMed: 12409470]
- It is noted that the product of 1b and 2a and NMR spectra of the product in aqueous solution at pH 7.4 were reported by Bandyopadhyay and Gao (2015).
- Kanichar D, Roppiyakuda L, Kosmowska E, Faust MA, Tran KP, Chow F, Buglo E, Groziak MP, Sarina EA, Olmstead MM, Silva I and Xu HH, Chem. Biodiver, 2014, 11, 1381–1397.
- Metola P, Chapin BM and Anslyn EV, in Boron: Sensing, Synthesis and Supramolecular Self-Assembly, The Royal Society of Chemistry, 2016, pp. 44–60.
- Groziak MP, Chen L, Yi L and Robinson PD, J. Am. Chem. Soc, 1997, 119, 7817–7826.
- Cal PM, Vicente JB, Pires E, Coelho AV, Veiros LF, Cordeiro C and Gois PM, J. Am. Chem. Soc, 2012, 134, 10299–10305. [PubMed: 22642715]
- Groziak MP and Robinson PD, Collect. Czech. Chem. Commun, 2002, 67, 1084–1094.
- Kalia J and Raines RT, Angew. Chem. Int. Ed, 2008, 47, 7523–7526.

17. Sarina EA, Olmstead MM, Kanichar D and Groziak MP, Acta Crystallogr. Sect. C, 2015, 71, 1085–1088.
18. Peters JA, Coord. Chem. Rev, 2014, 268, 1–22.

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Figure 1:
NMR spectra of **5b** in DMSO-d₆. A: Portion of the ¹H NMR spectrum illustrating the splitting pattern of the methylene protons (left panel) and ¹¹B NMR spectrum of the same sample (right panel). B: ¹H and ¹¹B NMR spectra of the solution after addition of D₂O. Full spectra are shown in ESI.

Figure 2.
X-ray structure of **5a** (left) and **5b** (right)

Figure 3.

Selected sections of ^1H NMR spectra of 2 mM solutions of **4b** at pH 9, 7, 6 and 4. Singlets from the imino protons of **3b** and **4b** and the aldehyde proton of **1a** are indicated. Two isomers are observed for **3b**. The full range of the ^1H NMR spectra as well as intermediate pH values are shown in ESI.

Figure 4.
The product of **1a** and **2a** in DMSO-d_6 before (A) and after (B) a drop of D_2O is added.

Figure 5 –.
¹H NMR spectrum of 7c in aqueous solution at pH 7 (A) and pH 4 (B).

Figure 6.
Crystal structure of **7c** (left) and **7e** (right)

Figure 7 –.

BSA conjugated to an α -amino hydrazide or an aliphatic hydrazide was allowed to react with an fPBA-fluorophore conjugate as described in ESI. After rapid gel filtration, the samples were analyzed by SDS-PAGE Lane 1: BSA + fluorophore (control). Lane 2: BSA- α -amino hydrazide + fluorophore. Lane 3: BSA-hydrazide + fluorophore. Top panel: Gel imaged under long wavelength UV light. Bottom panel: Coomassie stained gel.

Scheme 1.

Structure of a DAB and structures of *ortho*-carbonylphenylboronic acids and carbohydrazides.

Scheme 2.

Products of the reaction of **1a** with aliphatic carbohydrazides **2a** and **2b** in concentrated aqueous solution, isolated in solid form.

Scheme 3.

Products of the reaction of **1a** with aliphatic carbohydrazide **2b** in dilute aqueous solution.

Scheme 4.

Products of the reaction of **1a** or **1b** with α -amino hydrazides **2c** or **2d** in aqueous solution.