Bioorthogonal Reactions

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Vinylboronic Acids as Fast Reacting, Synthetically Accessible, and Stable Bioorthogonal Reactants in the Carboni–Lindsey Reaction

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Abstract: *Bioorthogonal reactions are widely used for the chemical modification of biomolecules. The application of vinylboronic acids (VBAs) as non-strained, synthetically accessible and water-soluble reaction partners in a bioorthogonal inverse electron-demand Diels–Alder (iEDDA) reaction with 3,6-dipyridyl-s-tetrazines is described. Depending on the substituents, VBA derivatives give second-order rate constants up to 27* $M^{-1} s^{-1}$ *in aqueous environments at room temperature, which is suitable for biological labeling applications. The VBAs are shown to be biocompatible, non-toxic, and highly stable in aqueous media and cell lysate. Furthermore, VBAs can be used orthogonally to the strain-promoted alkyne–azide cycloaddition for protein modification, making them attractive complements to the bioorthogonal molecular toolbox.*

In the last decades, the development of selective reactions between two reactants that are unaffected by any of the naturally occurring biological functionalities has been a major research area in chemical biology.[1] These bioorthogonal reactions (Figure 1 A) make it possible to chemically modify biomolecules in their native cellular environment and gain a better understanding of their role in a specific biological system or process. The bioorthogonal reaction should be high yielding and rapid, and the reactants and product(s) should be soluble and stable in aqueous media and non-toxic to the biological system. The use of small reactants is preferred to minimize steric interactions with the biomolecule or to facilitate incorporation by the endogenous cellular machinery.

The Carboni–Lindsey (CL) reaction between an electron-poor tetrazine (Figure 1 B, **I**) and an alkene has gained considerable attention for use in bioorthogonal applications.[2] As linear and unmodified alkenes (Figure 1 B, **VI**) showed only poor reaction rates with tetrazines,[3] most research has been directed to the development and use of strained alkenes such as *trans*-cyclooctene (TCO),[4] norbornene,[5] cyclopropene,[6] or *N*-acylazetine[7] (Figure 1 B, **II**–**V**). In addition to strain, the rate of the CL reaction can be significantly enhanced by the introduction of electron-donating substituents on the alkene bond.[8] This aspect, however, has been poorly investigated in relation to bioorthogonal applications.

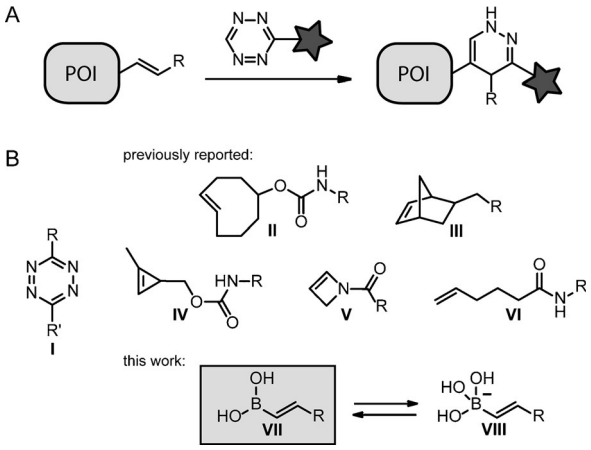

Figure 1. A) Labeling of the protein of interest (POI) using the CL reaction. B) Reagents of the CL reaction. Tetrazine **I**, *trans*-cyclooctene **II**, norbornene **III**, cyclopropene **IV**, *N*-acylazetine **V**, terminal alkene **VI**, vinylboronic acid **VII**, and the borate form **VIII**.

Vinylboronic acids (Figure 1 B, **VII**) are an interesting class of compounds with unique electronic properties, which are due to their vacant p-orbital. As a result of the inductive effect that is caused by the electronegativity difference of boron and carbon, the electronic deficiency of boron and the electron-donating oxygens attached to boron, boronic acid is considered to be a weak electron-donor.[9] Furthermore, boronic acids are mild organic Lewis acids, which in basic aqueous media are in equilibrium with their boronate anion (Figure 1 B, **VIII**), which is a strong electron-donor.[9] Although Diels–Alder reactions are known to proceed faster in aqueous media, the CL reactions of vinylboronic acids and alkylboronic acids are reported only in organic solvents, and high temperatures were often needed for the reaction to proceed.[10]

In our effort to investigate the electronic effects of the alkenes in the CL reaction, we found that vinylboronic acids (VBAs) show impressive reaction rates with 3,6-dipyridiyl-*s*-tetrazines in aqueous environments. Herein, we evaluate VBAs as synthetically accessible, fast reacting bioorthogonal reaction partners with 3,6-dipyridyl-*s*-tetrazines. We show that VBAs are stable, non-toxic, and can be used for protein labeling in vitro and in cell lysate. The hydrophilic properties and the small size of VBAs make them attractive for use in

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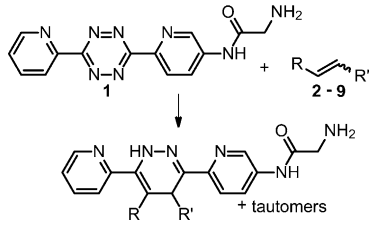
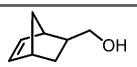
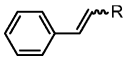
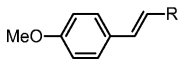
protein modifications and other biomolecular labeling applications.

To explore the potential of VBAs as bioorthogonal reaction partners, we first examined the second-order rate constants (k_2) of 3,6-dipyridyl-*s*-tetrazine derivative **1** with several VBAs in 5% MeOH in PBS (Table 1 and the Supporting Information, Figures S1 and 2) and compared them to the k_2 of norbornene **2**, a reagent that is used extensively in bioconjugation reactions.[2,5] Vinylboronic acid **3** reacted with tetrazine **1** with a rate of 3.0 $M^{-1}s^{-1}$, which is comparable to the rate of **1** with norbornene **2**. A methyl substituent on the alkene reduced the reaction rate, although the rate of the (*Z*)-isomer was almost 3-fold faster than that of the (*E*)-isomer of **4**. Addition of a phenyl ring to the VBA increased the k_2 significantly, (*E*)-phenylvinylboronic acid **5** showed a five-fold higher rate constant than norbornene **2**, comparable to the reported rate constant of the fastest cyclopropene.[6] For this alkene, the (*Z*)-isomer of **5** showed a lower rate constant than (*E*)-**5**. Introduction of an electron-donating substituent on the phenyl ring of **8** increased the rate constant further to 27 $M^{-1}s^{-1}$.

Table 1: Second-order rate constants of tetrazine **1** with several alkenes.



	alkene	No.	R	k_2 [$M^{-1}s^{-1}$]
1		**2**[a]	–	2.2 ± 0.1
2		**3**	$B(OH)_2$	3.0 ± 0.1
3		(*E*)-**4**	$B(OH)_2$	0.24 ± 0.01
		(*Z*)-**4**	$B(OH)_2$	0.65 ± 0.02
		(*E*)-**5**	$B(OH)_2$	11 ± 1
4		(*Z*)-**5**	$B(OH)_2$	0.59 ± 0.03
		6	H	< 0.025[c]
		(*E*/*Z*)-**7**[b]	OMe	< 0.025[c]
5		**8**	$B(OH)_2$	27 ± 2
		9	H	0.13 ± 0.01

[a] Endo/exo = 2:1. [b] *E*/*Z* = 1:3. [c] k_2 could not be determined accurately, owing to the slow reaction rate and low solubility of the alkenes in water.

To evaluate the effect of the boronic acid on the alkene's reactivity, we compared the rate constants of the fastest VBAs (*E*)-**5** and **8** to the alkene derivatives lacking the boronic acid (Table 1, Entries 4 and 5). Under the selected conditions, we could estimate a decrease in reaction rate of styrene **6** and *p*-methoxystyrene **9** of at least two orders of magnitude, although because of the reduced reaction rate and low solubility of the unsubstituted alkene in the aqueous environment, we could not determine the k_2 accurately. Introduction of an electron-donating alkoxy substituent on the alkene as in β-methoxystyrene **7** did not result in a significant increase in reaction rate, clearly indicating that the boronic acid has a large positive effect on the reactivity of the alkene. A similar trend in the rate constants of the alkenes **2**–**9** with 3,6-dipyridyl-*s*-tetrazine **10** was observed in 50% MeOH/PBS, although the constants decreased significantly in these less aqueous conditions (Supporting Information, Table S1 and Figures S3 and 4).

The reactions of VBAs **3**, (*E*)-**5**, and **8** were additionally studied with 3-phenyl-*s*-tetrazine and 3-phenyl-6-methyl-*s*-tetrazine (Supporting Information, Figure S5). Interestingly, compared to the reaction rate of these tetrazines with norbornene **2**, the reactions of the VBAs with the tetrazines are much slower than expected, which shows that the VBAs are especially suitable in the CL reactions with dipyridyl-*s*-tetrazines.

We then studied the reaction of 3,6-dipyridyl-*s*-tetrazine **10** and the most reactive VBA **8** in more detail (Figure 2A). We observed quantitative formation of dihydropyradizine **11** as the most abundant isomer, which lacked the boronic acid.

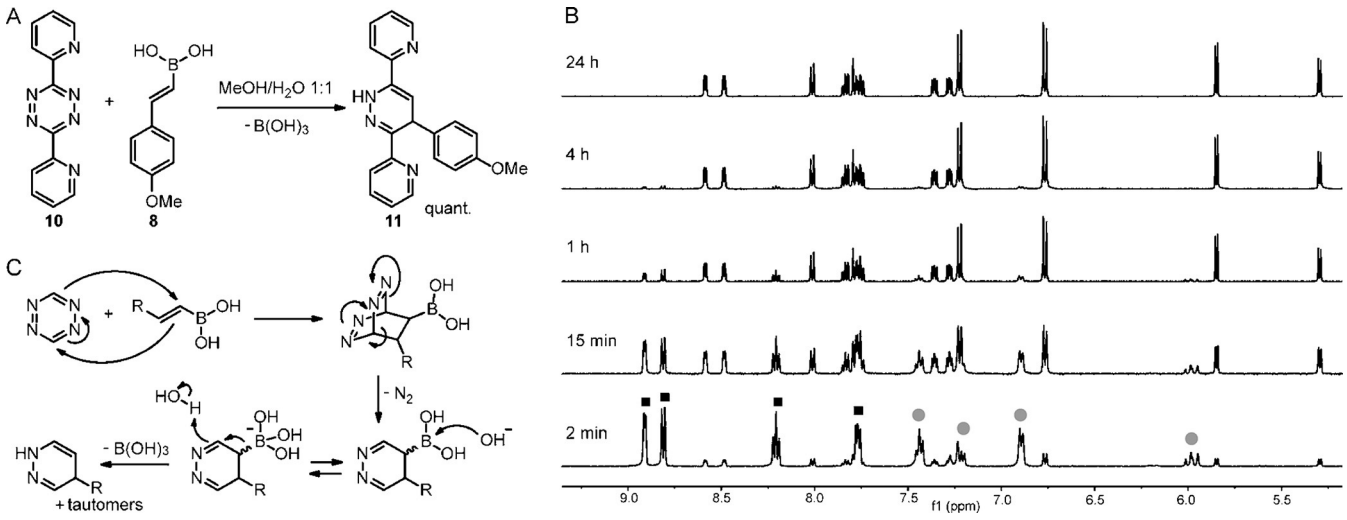


Figure 2. The reaction of tetrazine with vinylboronic acids A) Scheme of the reaction of 3,6-dipyridyl-*s*-tetrazine **10** with VBA **8**. B) ^{1}H NMR study of the reaction between 3,6-dipyridyl-*s*-tetrazine **10** (■) and VBA **8** (●). C) Proposed reaction mechanism.

No intermediates were observed in the ^{1}H NMR spectra during the reaction between **10** and **8**, which shows that the protodeborylation of boric acid is fast (Figure 2B and the Supporting Information, Figure S6). We therefore propose a mechanism in which boric acid is released immediately after the iEDDA cycloaddition, followed by dinitrogen release by a retro-Diels–Alder reaction (Figure 2C). The dihydropyridazine product **11** is the same as found in the reaction of tetrazine **10** with *p*-methoxystyrene **9** (Supporting Information, experimental section),[11] and slow isomerization and oxidization to the pyridazine product[8b] were observed by ^{1}H NMR and UV/Vis absorption (Supporting Information, Figures S7–9).

Next, we examined the biocompatibility of the VBAs using the most reactive alkene **8**. In the presence of most common functional groups such as amines, alcohols, carboxylic acids, and thiols, **8** was stable for at least seven days as observed by ^{1}H NMR (Supporting Information, Figure S10). Additionally, we incubated VBA **8** in cell lysate up to 24 h before addition of tetrazine **1** and observed only marginal changes in the reaction rate, which indicates that **8** was stable in the presence of all biomolecules in the cells (Supporting Information, Figure S11). For possible future applications of VBAs in live cells, we assessed the toxicity of VBA **8**, tetrazine **1**, boric acid, the side product of the CL reaction, and the dihydropyridazine product on NIH-3T3 cells. No toxicity was observed after 24 h for concentrations up to 100 μM for VBA **8** and boric acid, whereas tetrazine **1** and the dihydropyrazine product showed some toxicity at this concentration after 24 h (Supporting Information, Figure S12).

The CL reaction between VBAs and 3,6-dipyridiyl-*s*-tetrazine was next evaluated for its use for in vitro protein modification. Vinylboronic acids can be easily synthesized as the protected ester in one step by, for example, cross-metathesis between an alkene and a vinylboronic ester[12] or hydroboration of an alkyne.[13] Hydrolysis of the boronic ester occurs spontaneously in aqueous media, although many deprotection strategies to the boronic acid are also available.[9] For the uniformity of this work, we initially synthesized the azide-containing VBA-probe **13**, containing a free boronic acid (Figure 3A and the Supporting Information, experimental section), which was obtained by hydroboration of a phenylacetylene derivative followed by removal of the ester through the conversion and hydrolysis of the organotrifluoroborate.

We equipped a model protein human serum albumin (HSA), which contained one free cysteine, with a dibenzocyclooctyl (DBCO) function using commercially available DBCO-maleimide **12** (Figure 3B and the Supporting Information, Figure S13). The HSA-DBCO was then reacted with **13**, demonstrating that VBAs can be used orthogonally to the strain-promoted alkyne–azide cycloaddition (SPAAC) between a DBCO and an azide function. Notably, the VBA is not orthogonal to a Cu^I-mediated reaction between an

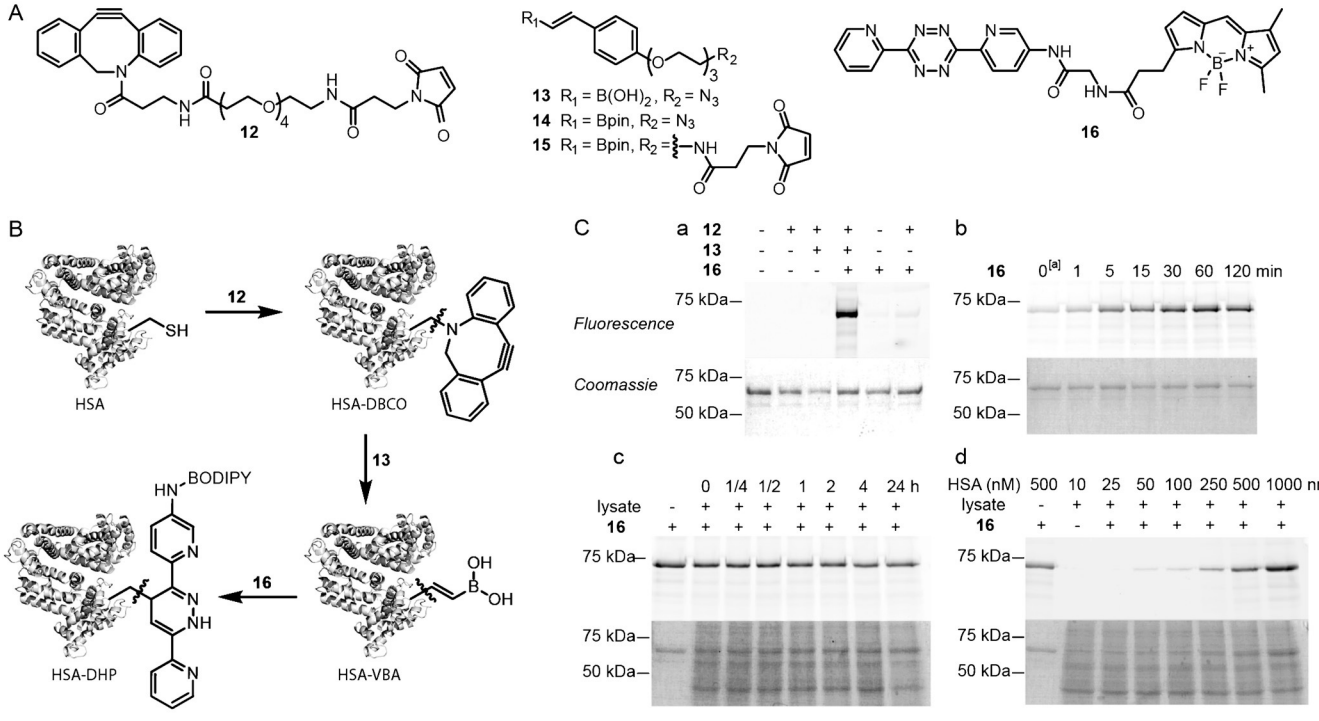


Figure 3. Evaluation of the CL reaction with HSA functionalized with VBA probe **13**. A) The structure of the molecules used for the protein modifications. B) The route used for individual HSA modification steps using **12**, **13**, and **16**. C) Fluorescent image (top) and Coomassie stain (bottom) of SDS-PAGE gels showing a) the individual modification steps of HSA (50 μM) as shown in B with **12**, **13**, and **16** (respectively 2, 10, and 10 equiv), b) the time range of the CL reaction of HSA-VBA (50 μM) and tetrazine **16** (10 equiv), c) the stability of HSA-VBA (0.5 μM) in cell lysate, and d) a concentration range of HSA-VBA followed by the CL reaction with tetrazine **16** (10 equiv) in cell lysate. [a] At $t = 0$ min, 1% labeling is expected because a premix of fluorescent tetrazine **16** and tetrazine **10** (1000 equiv) is added.

azide and an alkyne owing to copper-mediated boronic acid degradation.[14] We used tetrazine-BODIPY probe **16** (Figure 3A and the Supporting Information, experimental section) to visualize the VBA-modified HSA protein. Complete conversion to the dihydropyridazine (HSA-DHP) product was observed by mass spectrometry (Supporting Information, Figure S14), and the appearance of a green fluorescent band was observed by fluorescence imaging of the SDS-PAGE gel (Figure 3C, a).

As boronic esters are more synthetically accessible than the free boronic acids, we evaluated the stability of the pinacol boronic ester of **13** in aqueous media. Pinacol boronic ester **14** hydrolyzed to the boronic acid in PBS within 15 min (Supporting Information, Figure S15). We observed similar labeling results as with the free boronic acid **13**, which shows the suitability of vinylboronic esters as VBA precursors (Supporting Information, Figure S16). Additionally, we synthesized the VBA precursor **15**, which contained a maleimide for direct labeling of HSA. With VBA-maleimide **15**, similar labeling intensities were observed after the CL reaction with **16** as in the two-step approach described above using the SPAAC reaction (Supporting Information, Figure S17).

We evaluated the rate of the CL reaction and the stability of the HSA-VBA protein in cell lysate. Reaction of HSA-VBA with 10 equivalents tetrazine-BODIPY probe **16** provided a clear fluorescent signal on a SDS-PAGE gel within 5 min (Figure 3C, b). For this experiment we incubated HSA-VBA with **16** for the indicated time points before addition of a 100-fold excess of non-fluorescent tetrazine **10,** which accounts for the faint band observed at 0 min. In addition, incubation of 1% HSA-VBA (0.5 μM) in cell lysate (1 mg mL^{-1}) before the addition of the tetrazine-BODIPY probe **16** did not result in a significant loss of signal after 24 h (Figure 3C, c), which confirms the high stability of VBAs in the presence of biomolecules. Finally, a dilution series of HSA-VBA in cell lysate showed that we could visualize the modified HSA protein by fluorescence imaging in concentrations up to 50 nM. At this concentration, a clear HSA band could no longer be distinguished on the gel when stained with Coomassie (Figure 3C, d), indicating the sensitivity of the CL reaction under these conditions.

In summary, we have shown the use of vinylboronic acids as novel class of bioorthogonal reactants with 3,6-dipyridyl-*s*-tetrazines in an iEDDA reaction. Although boron-containing organic compounds are rarely found in nature, they are extremely suitable for chemical biological applications due to their hydrophilicity, stability, and low toxicity. Their use in cellular systems has increased over the past years as, for example, a protease inhibitor,[15] biosensor,[16] fluorescent sensor,[17] activating reagent[18] or reactant in a metal-catalyzed bioconjugation reaction.[19] Herein, we add VBAs as bioorthogonal reactants to the molecular toolbox. While the reaction rates of tetrazines with TCO[4] are still orders of magnitude faster, the use of VBAs may be advantageous due to their chemical properties, stability, and accessibility, while showing suitable rate constants that are required for modification and conjugation of biomolecules.

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