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.

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.

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 1A) 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 1B, I) and an alkene has gained considerable attention for use in bioorthogonal applications.[2] As linear and unmodified alkenes (Figure 1B, 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 1B, 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.

Vinylboronic acids (Figure 1B, 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 1B, VIII), which is a strong electron-donor.[10] 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.[11]

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-dipyridyl-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
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 \( k_j \) 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 \( k_j \) 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 \( \text{m}^{-1} \text{s}^{-1} \), 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 \( k_j \) 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 \( \text{m}^{-1} \text{s}^{-1} \).

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

| alkene | No. | R          | \( k_j \) [\( \text{m}^{-1} \text{s}^{-1} \)] |
|--------|-----|------------|-------------------------------------------|
| 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                                     |
|        | (Z)-5 | B(OH)\(_2\) | 0.59 ± 0.03                                |
| 4      | 6   | H          | <0.025\(^{[c]}\)                           |
| 5      | 8   | B(OH)\(_2\) | 27 ± 2                                     |
|        | (E/Z)-7\(^{[c]}\) | OMe | <0.025\(^{[c]}\)                        |

[a] Endo/exo = 2:1. [b] E/Z = 1:3. [c] \( k_j \) 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 \( k_j \) accurately. Introduction of an electron-donating alkoxyl substituent on the alkene as \( \beta\)-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-dipryridyl-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 dipyrldyl-s-tetrazines.

We then studied the reaction of 3,6-dipryridyl-s-tetrazine 10 and the most reactive VBA 8 in more detail (Figure 2A). We observed quantitative formation of dihydropyridazine 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-dipryridyl-s-tetrazine 10 with VBA 8. B) \(^1\)H NMR study of the reaction between 3,6-dipryridyl-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)\cite{11} and slow isomerization and oxidation 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 $\mu$m 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-dipyridyl-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 dibenzoclooctyl (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$^{1}$-mediated reaction between an...
azide and an alkyne owing to copper-mediated boronic acid degradation.\textsuperscript{[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 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 tetrabenzene-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 tetrabenzene 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)\textsuperscript{[15]} before the addition of the tetrabenzene-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-stetrazines 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,\textsuperscript{[16]} biosensor,\textsuperscript{[18]} fluorescent sensor,\textsuperscript{[17]} activating reagent\textsuperscript{[19]} or reactant in a metal-catalyzed bioconjugation reaction.\textsuperscript{[20]} Herein, we add VBAs as bioorthogonal reactants to the molecular toolbox. While the reaction rates of tetrabenzes with TCO\textsuperscript{[21]} 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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[1] For recent reviews, see: a) I. Nikic, E. A. Lemke, Curr. Opin. Chem. Biol. 2015, 28, 164; b) P. Schich, C. R. Bertozzi, Org. Biomol. Chem. 2014, 12, 9307; c) D. M. Patterson, L. A. Nazarova, J. A. Prescher, ACS Chem. Biol. 2014, 9, 592; d) M. F. Debets, J. C. M. van Hest, F. P. J. T. Rutjes, Org. Biomol. Chem. 2013, 11, 6439.
[2] J. Šečkuté, N. K. Devaraj, Curr. Opin. Chem. Biol. 2013, 17, 761.
[3] a) A. Niederwieser, A. K. Spáčel, L. D. Nguyen, C. Jungst, W. Reutter, V. Wittmann, Angew. Chem. Int. Ed. 2013, 52, 4265; Angew. Chem. 2013, 125, 4539; b) Y.-J. Lee, Y. Kurra, Y. Yang, J. Torres-Kolbus, A. Deiters, W. R. Liu, Chem. Commun. 2014, 50, 13085.
[4] M. L. Blackman, M. Royzen, J. M. Fox, J. Am. Chem. Soc. 2008, 130, 13518.
[5] N. K. Devaraj, R. Weissleder, S. A. Hilderbrand, Bioconjugate Chem. 2008, 19, 2297.
[6] a) J. Yang, J. Šečkuté, C. M. Cole, N. K. Devaraj, Angew. Chem. Int. Ed. 2012, 51, 7476; Angew. Chem. 2012, 124, 7594; b) D. M. Patterson, L. A. Nazarova, B. Xie, D. N. Kamber, J. A. Prescher, J. Am. Chem. Soc. 2012, 134, 18638.
[7] S. B. Engelsma, L. I. Willems, C. E. van Paaschen, S. I. van Kasteren, G. A. van der Marel, H. S. Overkleeft, D. V. Filipov, Org. Lett. 2014, 16, 2744.
[8] a) F. Thalhammer, U. Wallfahrer, J. Sauer, Tetrahedron Lett. 1990, 31, 6851; b) J. Sauer, D. K. Heldmann, J. Hetzenegger, J. Krauthan, H. Sichert, J. Schuster, Eur. J. Org. Chem. 1998, 2885.
[9] D. G. Hall, Boronic Acids, Preparation and Applications in Organic Synthesis Medicine and Materials, Wiley-VCH, Weinheim, 2011, pp. 1 – 31.
[10] a) E. Gomez-Bengoa, M. D. Helm, A. Plant, J. P. A. Harity, J. Am. Chem. Soc. 2007, 129, 2691; b) M. D. Helm, J. E. Moore, A. Plant, J. P. A. Harity, Angew. Chem. Int. Ed. 2005, 44, 3889; Angew. Chem. 2005, 117, 3957; c) G. Seitz, F. Haenel, Arch. Pharm. 1994, 327, 673.
[11] J. W. Wijnen, S. Zavráří se, J. B. F. N. Engberts, M. Charton, J. Org. Chem. 1996, 61, 2001.
[12] For recent examples see: a) R. Hemelaere, F. Carreaux, B. Carbioni, J. Org. Chem. 2013, 78, 6786; b) E. T. Kiesewetter, R. V. O’Brien, E. C. Yu, S. J. Meeck, R. R. Schro, A. H. Hovyeda, J. Am. Chem. Soc. 2013, 135, 6026; c) B. Marcincic, M. Jankowska, C. Pietraszuk, Chem. Commun. 2005, 663; d) C. Morrill, R. H. Grubbs, J. Org. Chem. 2003, 68, 6031.
[13] R. Barbeyron, E. Benedetti, I. Cosy, J. J. Vasseur, S. Arseniyadis, M. Smietana, Tetrahedron 2014, 70, 8431.
[14] C. Dai, Y. Cheng, J. Cui, B. Wang, Molecules 2010, 15, 5768.
[15] R. Smoun, A. Rubinstein, V. M. Dembitsky, M. Srebnik, Molecules 2012, 112, 4156.
[16] A. R. Lippert, G. C. van de Bittner, C. J. Chang, Acc. Chem. Res. 2011, 44, 793.
[17] T. L. Halo, J. Appelbaum, E. M. Hobert, D. M. Balkin, A. Schepartz, *J. Am. Chem. Soc.* 2009, 131, 438.

[18] J. Kim, C. R. Bertozzi, *Angew. Chem. Int. Ed.* 2015, 54, 15777; *Angew. Chem.* 2015, 127, 16003.

[19] C. D. Spicer, T. Triemer, B. G. Davis, *J. Am. Chem. Soc.* 2012, 134, 800.

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