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Rapid Nickel(II)-Promoted Cysteine *S*-Arylation with Arylboronic Acids

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S-Arylation of cysteine residues is an increasingly powerful tool for site-specific modification of proteins, providing novel structure and electronic perturbation. The present work demonstrates an operationally-simple cysteine arylation reaction 2-nitro-substituted arylboronic acids, promoted by a simple nickel(II) salt. The process exhibits strikingly fast reaction rates under physiological conditions in purely aqueous media with excellent selectivity toward cysteine residue. Cysteine arylation of natural proteins and peptides allows attachment of useful reactive handles for stapling, imaging, or further conjugation.

Chemical modification of biomacromolecules plays a prominent role in both fundamental chemical biology research and important industrial applications, such as biopharmaceuticals.[1–4] Bioconjugation allows chemists to build hybrid structures that combine attributes of biomacromolecules and small molecules or nonbiological polymers. Furthermore, chemical manipulation allows introduction of biorthogonal handles, ultimately addressing the remarkable challenge of designing chemo- and regio-selectivity in a sea of complex functional groups under biocompatible condition. Cysteine remains the most important target residue. Cysteine occurs with low abundance in proteins,[5] and exists in both reduced sulfhydryl and oxidized disulfide forms, each with its own unique chemistry. Cysteine alkylation classically focused on maleimide or α-haloacetamides and other electrophiles. Recent years have seen a proliferation of new approaches, including S_N2 reactions,[6–8] S_NAr reactions,[9–13] and metal-mediated reactions.[14–19] In many cases, new reactivity has been successfully adapted to rather complex applications.[20–23]

S-Arylation is a relatively new tool for cysteine modification.[9–12] Nucleophilic substitution (S_NAr) of aromatic or heteroaromatic electrophiles can be effective for this purpose.[24] The interactions of transition metals with peptides and proteins[25,26] offers attractive alternatives approach to bioconjugation,[27–30] and cross-coupling approaches to S–H or N–H arylation are among the most attractive and potentially valuable approaches. In practice, however, traditional cross-coupling for cysteine arylation remains limited,[17,18,31] and the community has recently developed an alternative approach with highly reactive organometallic reagents.[14–16,32] Optimization of ligand structure led to remarkably selective and rapid cysteine modification, even in quite complex proteins, with arylpalladium[16,32] and (quite recently) arylgold[14,15] reagents.

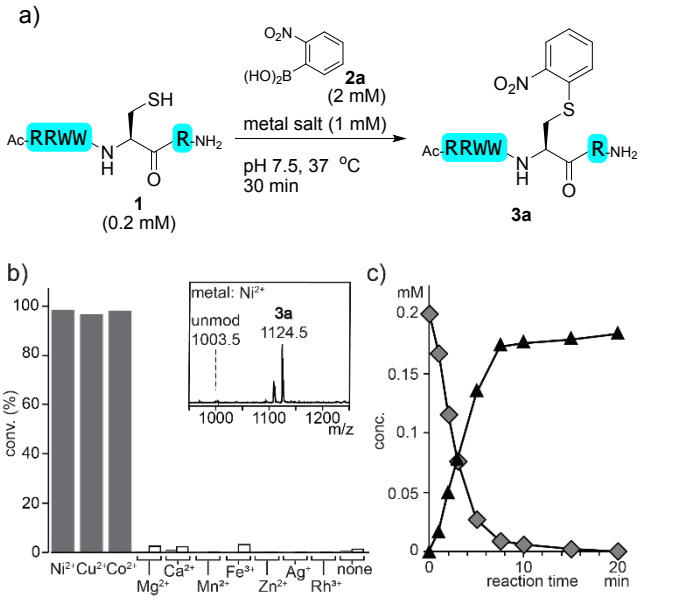

Figure 1. (a) Modification of IL-8 inhibitor (**1**) with boronic acid **2a**. Condition: **1** (0.2 mM), **2a** (2 mM), and metal salt (1 mM) in *N*-methylmorpholine (NMM) buffer (10 mM, pH 7.5) at 37 °C for 30 min. (b) Conversion in the presence of various metal salts after incubation for 30 min (solid bar): $Ni(OAc)_2$, $Cu(OAc)_2$, $Co(OAc)_2$, $Mg(OAc)_2$, $CaCl_2$, $Mn(OAc)_2$, $Fe(NO_3)_3$, $Zn(OAc)_2$, AgOAc and $RhCl_3$,. For Mg(II), Ca(II), Mn(II), Fe(III), Zn(II), Ag(I), and Rh(III), reaction time was elongated to 24 h (plain bar). Conversion calculated from MALDI-MS of crude reaction mixture. Inset: crude MALDI-MS spectrum Ni^{2+}. An additional peak [M(**3a**)−16] derived from oxygen atom loss from the nitro group was observed. (c) Kinetic data of Ni^{2+}-promoted reaction of **1** with **2a** by RP-HPLC; **1** (shaded rhombus) and **3a** (solid triangle).

We have recently become interested in boronic-acid–based bioconjugation technologies.[33–36] Boronic acids are attractive reagents in biological chemistry and in bioconjugation specifically, because they have good aqueous stability and

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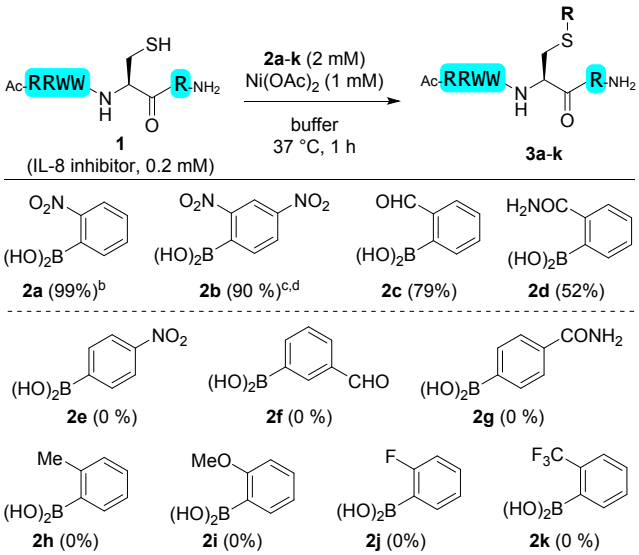
solubility, yet exhibit novel chemistry distinct from naturally-occurring functional groups. Herein, we report a class of boronic acids that exhibit exquisitely selective and rapid cysteine arylation in reactions promoted by Ni^{2+}.

We screened a wide variety of metal ions as potential promoters of bioconjugation reactions with simple arylboronic acid reagents, and no cysteine reactivity was observed in any reaction. However, in the course of structure-reactivity studies, we have discovered that 2-nitrophenylboronic acid reagents exhibit novel metal-promoted cysteine arylation. We examined the reaction of IL-8 inhibitor (**1**) containing a single cysteine residue as a model peptide with 2-nitrophenylboronic acid (**2a**) in the presence of a variety of metal salts. Quantitative conversion was observed for nickel(II), copper(II), and cobalt(II) after 30 min at pH 7.5 (Figure 1b). MALDI-TOF MS/MS fragmentation analysis of product verified that the modification reaction occurred on the cysteine residue (Figure S1). The chemical structure of the *S*-arylated product was confirmed by ^{1}H NMR for the reaction of *N*-acetylcysteinamide as a model substrate (Figure S2).

Table 1 Scope of boronic acid[a]



[a] Modification reaction conditions: IL-8 inhibitor (**1**) (0.2 mM), boronic acid **2a–k** (2 mM), and $Ni(OAc)_2$ (1 mM) in *N*-methylmorpholine (NMM) buffer (10 mM, pH 7.5) at 37 °C for 1 h. Relative conversion shown in the parenthesis was assessed via MALDI-TOF MS of crude reaction mixture. [b] Reaction time was 15min. [c] Reaction was carried out in MES buffer (10 mM, pH 6.5) at 37 °C for 30 min. [d] 46% conversion under the same conditions without $Ni(OAc)_2$.

The reaction kinetics are remarkably fast: the reaction half-life was <4 minutes, and conversion >95% was observed within ten minutes at pH 7.5 in the presence of 1 mM Ni^{2+} (Figure 1c, Figure S3, and Figure S4). To allow comparison with other benchmarks under operationally relevant conditions,[37,38] we calculated an apparent second-order rate constant, k_{app} ~1.6 $M^{-1}s^{-1}$ (where $k_{app} = k^1/[\mathbf{2a}]$) for modification of IL-8 inhibitor **1** under pseudo first-order conditions. By the strict definition of the term—a substance increasing reaction rate without undergoing any chemical change—Ni^{2+} is a catalyst for this transformation. Indeed, substoichiometric levels of Ni^{2+} (50 µM, 25 mol%) also mediate *S*-arylation, albeit with significantly diminished reaction rate (Figure S5). We chose nickel-promoted arylation for further development due to its superior reaction kinetics and to avoid the potential for competing histidine-directed amide N–H modification known for copper. Nickel –promoted reactions also avoid potential copper-induced cysteine oxidation.[39]

Table 2 Peptide screens[a]

entry	sequence	convn (%)
1	Ac-RRWW**C**R-NH_2 (**1**)	>99
2	Ac-RRWW**C**HRK-NH_2 (**4**)	98
3	H-MSRPA**C**PNDKYE-OH (**5**)	>99
4	H-**C**GHGNKSGLMVGGVV-OH (**6**)	>99
5	H-**C**YIQN**C**PLG-NH_2 (C^1-C^5)[b] (**7**)	91[c]
6	H-AG**C**KNFFWKTFTS**C**-OH (C^3-C^{13})[b] (**8**)	>99[d]
7	H-DRVYIHPFHL-OH (**9**)	—
8	H-SFLLRN-NH_2 (**10**)	—
9	H-YGRKKRRQRRR-OH (**11**)	—
10	H-GNLWATGHFM-NH_2 (**12**)	—

[a] Modification reaction conditions: Peptide (0.2 mM), **2a** (2 mM), and $Ni(OAc)_2$ (1 mM) in NMM buffer (10 mM, pH 7.5) at 37 °C for 30 min. Relative conversion was assessed via MALDI-TOF MS of crude reaction mixture. [b] The reaction was carried out after treatment with TCEP (0.8 mM) at 37 °C for 30 min. [c] Product ratio: mono-/di-arylated peptide, 1:1.6. [d] Product ratio: mono-/di-arylated peptide, 1:3.8.

The modification reaction exhibits a unique boronic acid scope (Table 1 and Figure S6). Boronic acids with electron-deficient substituents in the 2-position are required for reactivity (**2a–d**). Regioisomers in the meta or para positions were unreactive (**2e–g**), as were boronic acids with electron-donating substitution (e.g. **2h** and **2i**). The very electron-deficient 2,4-dinitrophenyl reagent **2b** was also reactive under these conditions, but reagents with purely σ-electron-withdrawing groups, without an electron-deficient π system, such as fluoro (**2j**) and trifluoromethyl (**2k**) were unreactive. Reactivity was observed with the carboxamide-substituted reagent **2d**, which has limited electron-withdrawing power, albeit at decreased reaction rate and conversion. It is worth noting that the observed reactivity trends differ significantly from other reports: electron-poor arylboronic acids are typically sluggish in Chan-Lam coupling.[40] Furthermore, nickel-promoted Chan–Lam coupling is rare,[41] and we are not aware of any examples of a cobalt-promoted process.

A variety of cysteine-containing peptides react quickly and efficiently under the nickel-promoted conditions (Table 2, entries 1–6). MS/MS analysis of arylated peptides revealed that modification occurred on cysteine even in the presence of competitive nucleophilic residues (Figure S7–S10). Oxidized disulfide sequences are unreactive under the nickel-promoted conditions, but pre-treatment with a reducing agent (TCEP) liberated a reduced thiol, and the reducing agent did not interfere with a subsequent modification reaction (entry 5,6).

The present method can be readily used to prepare *meta*-linked stapled peptides. Peptide stapling can increase stability against proteases and change both chemical and biological properties. Stapling of oxytocin, a disulfide bond-containing peptide hormone, with commercially available 4,5-difluoro-2-nitrophenylboronic acid (**13**) was examined, where initial nickel-promoted boronic-acid coupling would be expected to encourage facile intramolecular S_NAr to cyclize onto the second cysteine residue. Carrying out the reaction at slightly acidic pH served to minimize the formation of diarylated peptides. These conditions afforded a stapled oxytocin **14** (Figure 2 and S11). The product stapled peptide, containing a monofluorinated nitrophenyl moiety, displayed high chemical stability (Figure S12). It remained intact after incubation for 24 hours at pH 11, indicating the robust nature of the staple. Consistent with many examples of peptide stapling,[9,24,42,43,32] stapled oxytocin **14** displays significant stability in serum and protease stability assays, relative to peptide mixtures and to reduced acyclic oxytocin, respectively (Figures S13–S14).

While most modification products are serum-stable, extremely electron-deficient modifications (e.g. with dinitro-boronic acid **2b**) are reactive in thiol exchange reactions. A cysteine-containing peptide (**4**) modified with boronic acid **2b** reverts to the original peptide **4** upon treatment with 1,2-dithiothreitol (Figure S15), consistent with prior reports.[44] Thus, the present method also provides an alternative route to substrates for thiol-exchange reactions, and one that avoids selectivity issues that can arise in simple S_NAr reactions with electrophiles such as 1-fluoro-2,4-dinitrobenzene.[45]

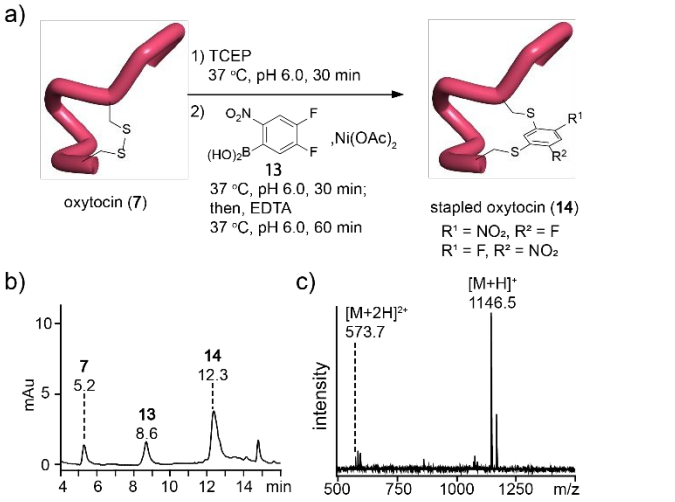


Fig. 2 (a) Peptide stapling of oxytocin (**7**) with 4,5-difluoro-2-nitrophenylboronic acid (**13**). Conditions: oxytocin (**7**) (0.2 mM), **13** (1 mM), and $Ni(OAc)_2$ (1 mM) in MES buffer (10 mM, pH 6) at 37 °C for 30 min, followed by further incubation at 37 °C for 60 min after addn of EDTA (8 mM). (b) HPLC analysis data of the crude reaction mixture. Numbers on each peak represent retention time (min). (c) MALDI-TOF MS spectrum of stapled oxytocin **14** after purification.

The reaction can be applied to protein substrates with high efficiency (Figure 3). Bovine serum albumin (BSA) contains a single reduced cysteine thiol, Cys34. To facilitate analysis of protein modification, we synthesized alkyne-functionalized 2-nitrophenylboronate **15b**, which allows quantitative detection of alkyne incorporation on a blot membrane by "chemical blotting" with 3-azido-7-hydroxycoumarin, a fluorogenic azide.[46] Modification of BSA under conditions developed for peptides was quite sluggish. To improve reaction efficiency, we screened several bipyridine-based ligand additives. A significant increase in reaction efficiency was observed with 6,6'-dimethylbipyridine (Figure 3a and S16). The role of the added ligand remains opaque, but it could function to limit nonspecific coordination to protein surface residues. The beneficial effect of bipyridine ligands is not observed in reactions of smaller peptide substrates.

Efficient modification of BSA with the 2-nitrophenylboronic acid **15b** was observed within 15 minutes, whereas no meaningful signal for product was observed in a negative control with boronic acid (**15a**) (Figure 3b and S17). The modification persisted after exposure to acidic, basic, and reductive conditions, as well as to glutathione (Figure S18). Consistent with a previous report,[16] a significant decrease in arylated BSA was observed upon overnight exposure to oxidants ($NaIO_4$ or H_2O_2). We also tested modification of a recombinant B-domain of *S. aureus* protein A (FBF6C) that contains a single cysteine thiol (Phe6Cys). LC–MS experiments demonstrate clean and complete conversion to a single cysteine-arylated product (Figure 3c and S19). Negative control experiments in the absence of nickel showed no modification for either BSA or FBF6C.

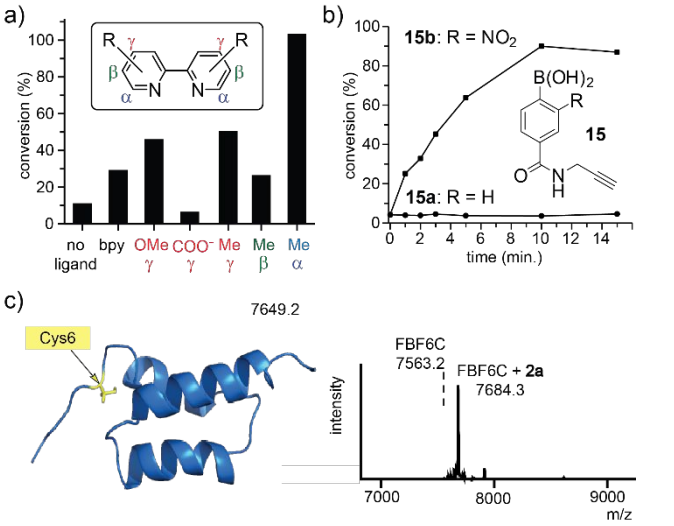


Fig. 3 Modification of proteins with 2-nitrophenylboronic acids. (a) Modification of BSA in the presence of bipyridine ligands. Alkyne incorporation measured by chemical blot. Conditions: BSA (50 μM), ligand (1 mM), boronic acid **15b** (0.5 mM), and $Ni(OAc)_2$ (1 mM) in NMM buffer (50 mM, pH 7.4) at 37 °C for 30 min. (b) Kinetics of BSA modification with **15a**, **15b**. Conditions: BSA (50 uM), 6,6'-dimethylbipyridine (1 mM), boronic acid (0.5 mM), and $Ni(OAc)_2$ (1 mM) in NMM buffer (50 mM, pH 7.4) at 37 °C. (c) Modification of FBF6C with boronic acid **2a**. Conditions: FBF6C (50 μM), boronic acid **2a** (0.5 mM), TCEP (250 μM), 6,6'-dimethylbipyridine (1 mM), and $Ni(OAc)_2$ (1 mM) in NMM buffer (50 mM, pH 7.4) at 37 °C for 30 min. LC–MS analysis of crude reaction. Met1 was not present in the isolated protein.

The nickel-promoted cysteine arylation reported here is a simple, rapid, and efficient protein modification tool. It produces stable bioconjugation products from cheap, stable,

and often commercially available reagents. An interesting ligand acceleration was observed, enabling cysteine arylation of natural proteins to incorporate useful reactive handles for stapling, imaging, or further conjugation.

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Conflicts of interest

There are no conflicts to declare.

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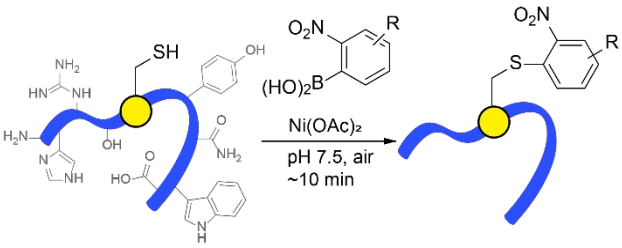

Nickel salts catalyze fast cysteine arylation with 2-nitroarylboronic acids. The process uses cheap, readily-available reagents and allows introduction of diverse chemical handles.