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Alkynone β-trifluoroborates: A new class of amine-specific biocompatible click reagents

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Amine-targeting reactions that work under biocompatible conditions or in water are green processes that are extremely useful for the synthesis of functional materials and biotherapeutics. Unfortunately, despite the usefulness of this reaction, there are very few good amine-specific click methods reported thus far. Here, we report an amine-specific click reagent using alkynone β-trifluoroborates as the electrophiles. These boron-containing alkynyl reagents exhibit extremely high chemoselectivity toward amines even in the presence of thiols. The resulting oxaboracycle products are bench-stable, displaying the reactivities of both organoborates and enaminones. Intrinsic advantages of this methodology include benign reaction conditions, operational simplicity, remarkable product stability, and excellent chemoselectivity, which satisfy the criteria of click chemistry and demonstrate the high potential in bioconjugation. Hence, this water-based chemical approach is also applicable to the modification of native amino acids, peptides, and proteins. Ultimately, the essential role of water during the reaction was elucidated.

INTRODUCTION

The development of the metal-free "click" technology that can work in water or under biocompatible reaction conditions is especially important in the areas of green chemistry and chemical biology (*1*). This powerful method has just been recognized with the 2022 Nobel prize in chemistry. During the past two decades, click reactions including the copper-catalyzed azide-alkyne cycloaddition developed by Sharpless (*2*, *3*) and Meldal (*4*) independently and the strain-promoted metal-free azide-alkyne cycloaddition by Bertozzi *et al.* (*5*, *6*) have been widely used for the synthesis of biotherapeutics due to their bioorthogonal properties. Since the aforementioned approaches need prefunctionalization of the biomolecules, such as proteins, great efforts have also been directed toward the development of chemical conjugation methods that can directly conjugate with biomolecules (*7*–*9*). Among them, amine conjugation reactions are highly sought after, as free amine groups are prevalently found in native amino acids, especially at the N terminus and lysine residues in most polypeptide chains (*10*).

Traditional amine conjugation reactions can be achieved by using electrophilic reagents such as activated esters (*11*), vinyl sulfones/sulfonamides (*12*–*14*), iso(thio)cyanates (*15*), and aldehydes (*16*–*18*). Although *N*-hydroxysuccinimide (NHS) esters (*19*) are the most frequently used reagents in amine conjugation to form amide bonds, the generation of NHS residues cannot be neglected. In recent years, other chemical tools have been available for amine ligation (*20*–*27*), including 2-methylenemalonate (*20*), azaphilones (*21*), diazonium compounds (*22*), and boronic acids (*23*–*25*). Taking into account the incorporation of additional boron functional handles, 2-formyl and 2-acetyl benzeneboronic acids (2-FBBA and 2-ABBA) first developed by Gois and coworkers stand out as attractive primary-amine conjugation reagents (Fig. 1A) (*28*). Up to now, various boron-containing drugs have been approved for clinical use (Fig. 1B), such as bortezomib, crisaborole, and tavaborole (*29*). There is also a considerable amount of attention paid to the boron-containing payloads in boron neutron capture therapy (*30*) and antibody-drug conjugates (*31*, *32*). Furthermore, π-conjugated boracycles, such as BODIPY, can act as the fluorescent probes in vivo (*33*, *34*). In the field of bio-click chemistry, the boronic acid units have been recognized to facilitate the imine formation including iminoboronates (*28*) and diazaborines (*35*). Nonetheless, boronic acids such as 2-FBBA are not truly amine specific owing to the side reactivity of iminoboronates toward the free thiol groups from N-terminal cysteines (*36*). In addition, two free hydroxy groups on boronic components have propensity to form covalent bonds with endogenous polyols such as fructose (*37*, *38*). These limitations have prompted us to explore the use of other boron-containing electrophiles that can be applied to the water-based amine-specific reactions.

Here, we report a simple and practical method of amine conjugation using alkynone β-trifluoroborates as the amine-specific click reagents (Fig. 1C). 1,4-Click conjugate addition between acetylene Michael acceptors and nucleophiles has already been exploited in many organic transformations (*39*–*41*). However, the problems associated with cross-reactivity with other nucleophiles, retro-Michael addition (*42*), and the requirement of external bases in organic solvents (*43*–*45*) are yet to be addressed, hampering their direct applications in bioconjugation of proteins. We envisage that the introduction of boron substituents at β position will enable us to tune the chemoselectivity and stability, preventing retro processes via coordination of the carbonyl group with the boron moiety (*46*), thereby overcoming the limitations of the existing methods. Previously, β-boryl alkynyl ketones were applied in the heterocycle synthesis such as pyrazoles (*47*) and pyrimidines

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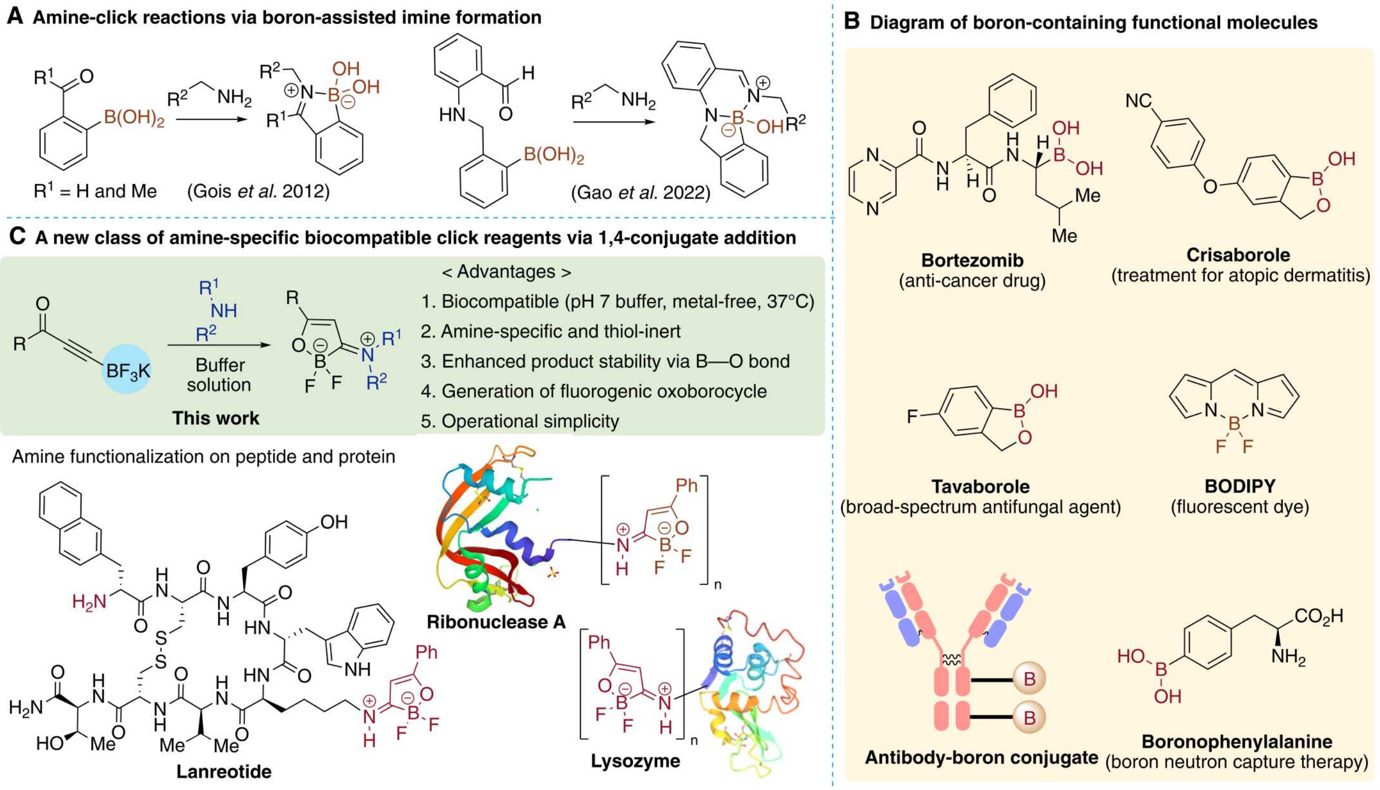


Fig. 1. Biocompatible click reaction. (**A**) Amine click reactions via boron-assisted imine formation. (**B**) Diagram of boron-containing functional molecules. (**C**) Amine-specific biocompatible click reagents by installing β-trifluoroborates onto alkynones (this work).

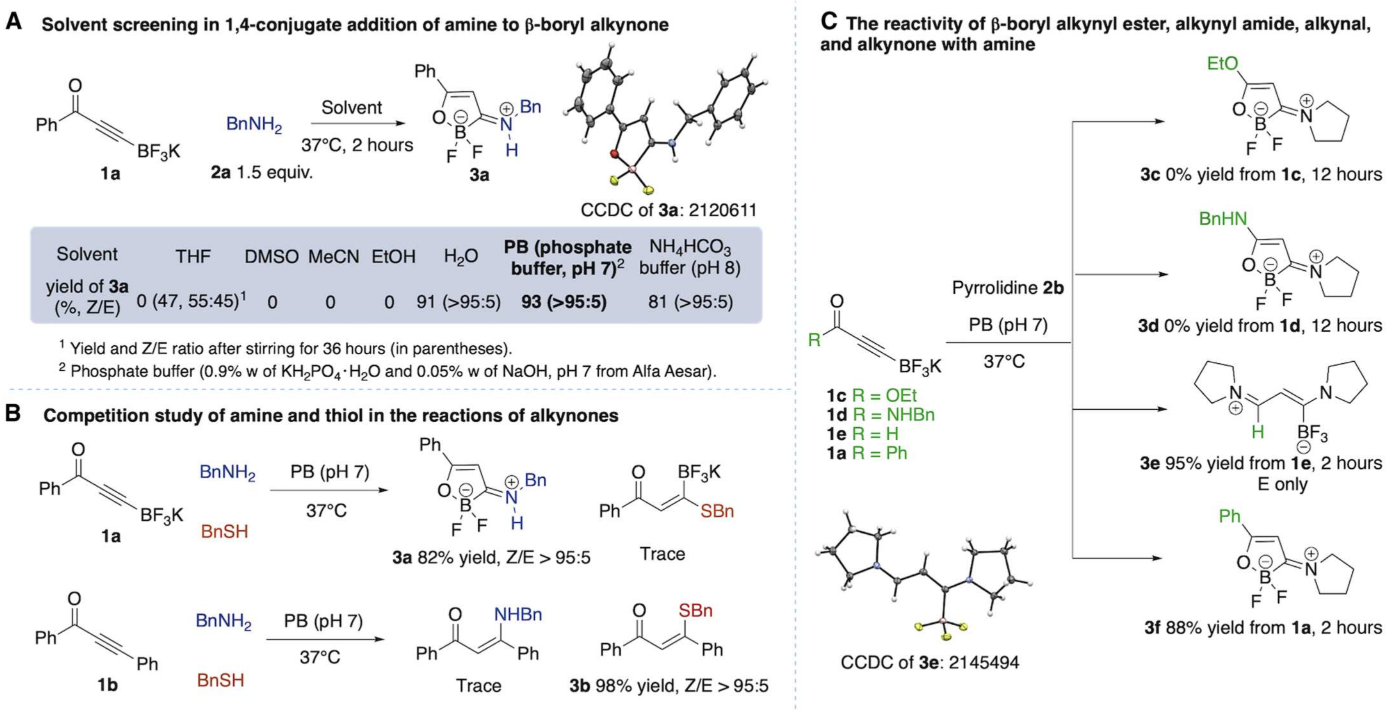
(*48*) from hydrazine and amidines, respectively. So far, no precedent has been reported in the direct conjugate addition of simple amines or amino biomolecules to alkynyl carbonyl β-borates, especially in water or under biocompatible conditions. It will be of great value to the family of biocompatible boron-containing building blocks in amine conjugation as the organic trifluoroborate salts are easy to synthesize and handle, exhibiting superior air, moisture, and polyol stability (*49*). For example, Zawada *et al.* (*50*) developed the arylethynyltrifluoroborates for the inverse electron demand Diels-Alder reactions with dipyridyl-tetrazines under physiological conditions. The feasibility of this process using an unstrained dienophile was attributed to the defluorination triggered by $AlCl_3$ and the subsequent formation of a strong boron-nitrogen bond. Furthermore, the pioneering findings by Molander *et al.* (*51*) and the exhaustive work by Kirkham, Harrity, and coworkers (*52*) demonstrate the high utility of organo-trifluoroborates in organic synthesis.

RESULTS

Reaction condition optimization

With our continuing interest in green chemistry (*53*–*56*) and the aim of harnessing the great potential of boron-containing alkynyl reagents in amine conjugation, we decided to embark on a study of the reaction of 1-phenylprop-2-ynone-3-trifluoroborate salt **1a** with benzylamine under metal-free conditions. In a preliminary study, the amine-conjugation process was observed at a sluggish rate when tetrahydrofuran was chosen as the solvent (Fig. 2A). In contrast, the reaction did not work in other organic solvents including dimethyl sulfoxide, acetonitrile, and ethanol. Next, we paid our attention to water-based solvent systems. Fortunately, the electrophile was found to be fully consumed within 2 hours in both deionized water and pH-neutral buffer media, affording a single product as a white precipitate (fig. S1). On the basis of the x-ray crystallography analysis, an unexpected five-membered oxaboracycle was generated to enhance the stability of the amine conjugate. Moreover, product **3a** was obtained predominantly in a Z configuration under the water-based conditions. To assess the orthogonality of this reaction, a competition study of benzylamine and benzyl mercaptan in a buffer solution of **1a** was carried out (Fig. 2B). Only the amine addition product was formed, indicating that the alkynone β-trifluoroborate functioned as a potent amine-specific click reagent. In addition, **1a** was found inert toward thiols in a water-based solution even in the absence of amine (fig. S21D). Alkynones without boron functionality such as 1,3-diphenylprop-2-ynone **1b** can also react with amine under the same conditions; however, the amine specificity of **1b** was sharply interfered when both amine and thiol were present (fig. S21, A and B). The likely involvement of the deprotonated, less dominant BnS^- anion (*57*) in the reaction of BnSH precludes straightforward comparisons of the reactivity of $BnNH_2$ and BnSH. Nevertheless, density functional theory (DFT) calculations show that the BnS^- anion, once generated, is indeed reactive, especially toward **1b** (see the Supplementary Materials for more details and discussion).

To investigate the role of the R group, reactions of β-boryl alkynone, alkynyl ester, alkynyl amide, and alkynal (**1a**, **1c** to **1e**) were



Solvent	THF	DMSO	MeCN	EtOH	H_2O	PB (phosphate buffer, pH 7)[2]	NH_4HCO_3 buffer (pH 8)
yield of 3a (%, Z/E)	0 (47, 55:45)[1]	0	0	0	91 (>95:5)	**93 (>95:5)**	81 (>95:5)

[1] Yield and Z/E ratio after stirring for 36 hours (in parentheses).

[2] Phosphate buffer (0.9% w of $KH_2PO_4 \cdot H_2O$ and 0.05% w of NaOH, pH 7 from Alfa Aesar).

Fig. 2. The screening of solvents and β-boryl electrophiles in the amine-specific biocompatible reaction. (**A**) Solvent effect. (**B**) Competition study of amine and thiol. (**C**) Reactivity evaluation of β-boryl alkynone, alkynyl ester, alkynyl amide, and alkynal.

performed with pyrrolidine **2b** under the optimal conditions (Fig. 2C). Only the alkynone β-trifluoroborate **1a** underwent 1,4-conjugate addition with pyrrolidine, affording **3f** in 88% yield within 2 hours. No desired product was detected in the reactions of alkynyl ester **1c** and alkynyl amide **1d** owing to the attenuated electrophilicity. The reactive electrophile, alkynal β-trifluoroborate, delivered the exclusive double-amine conjugate **3e**, which was determined by x-ray crystallography analysis.

Substrate scope

With the boron-containing alkynyl reagent **1a** and the optimal conditions in hand, we explored the substrate scope of amines in 1,4-conjugate addition (Fig. 3A). Both electron-deficient and electron-rich substituents on the phenyl ring of the amines were tolerable (**3g** to **3i**). The α-branched substituent on the benzylamine substrate had some influence on the reactivity, which required a longer reaction time for complete conversion. For cyclic and acyclic secondary amines, the reactions proceeded rapidly to provide **3c** and **3k** to **3m**. The structure of morpholine adduct **3l** was confirmed by x-ray crystallography analysis. Both propargylic amine and primary aliphatic amines could be effectively converted into the corresponding products **3n** to **3q** with a high Z/E ratio in most cases. We further examined 1,4-conjugate addition using aromatic amines. Aniline and other aryl amines having electron-rich groups on the arene moiety smoothly participated in the reaction, generating products with extended π-conjugated systems (**3r** to **3v**). For example, the indoline adduct **3v** was obtained in good yield, which exhibited strong fluorescence emission in a tetrahydrofuran (THF) solution (fig. S31). Next, the feasibility of other β-boryl alkynones was also tested (Fig. 3B). The alteration of the electronic character and the position of substituents on the electrophile's benzene ring did not compromise the efficiency of the process (**4a** to **4d**). More sterically hindered 2-naphthyl and 1-pyrenyl alkynones also worked well (**4e**, **4h**). The incorporation of an amide bond on the aromatic ring was viable in this reaction (**4c**). When the aryl ketone was replaced by the aliphatic counterpart, the formation of iminium trifluoroborates **5a** to **5c** via the double addition of amine outcompeted the expected ring-closure pathway regardless of the stoichiometric ratios and the number of substituents on substrates (primary/secondary amines and primary/secondary aliphatic ketones).

Product transformations and potential in bioconjugation

Next, we turned our attention to the applicability of the boron conjugates. Owing to the resonance form of enaminone and the boron functionality present in these molecules, the five-membered oxaboracycles can serve as versatile building blocks for subsequent transformations (Fig. 4A). For example, **3a** readily underwent the Suzuki-Miyaura reaction under the palladium-catalyzed conditions, affording the arylation product **6a** with an exclusive Z configuration (*58*). Besides the cross-coupling reaction, treatment of **3a** with a strong oxidant such as $NaBO_3 \cdot 4H_2O$ resulted in the formation of amide **6b**. The enaminone character in **3a** was demonstrated by the nucleophilic aromatic substitution on the highly electrophilic benzyne intermediate (*59*). The C-arylation of **3a** took place under the transition metal–free conditions, furnishing the fully substituted product **6c** in satisfactory yield. On the other hand, the formation of five-membered oxaboracycles provided a promising fluorophore motif. For example, the absorption and fluorescence spectra of pyrene-containing reagent **1h** and its corresponding adduct **4h** were measured and compared (Fig. 4B). We noticed

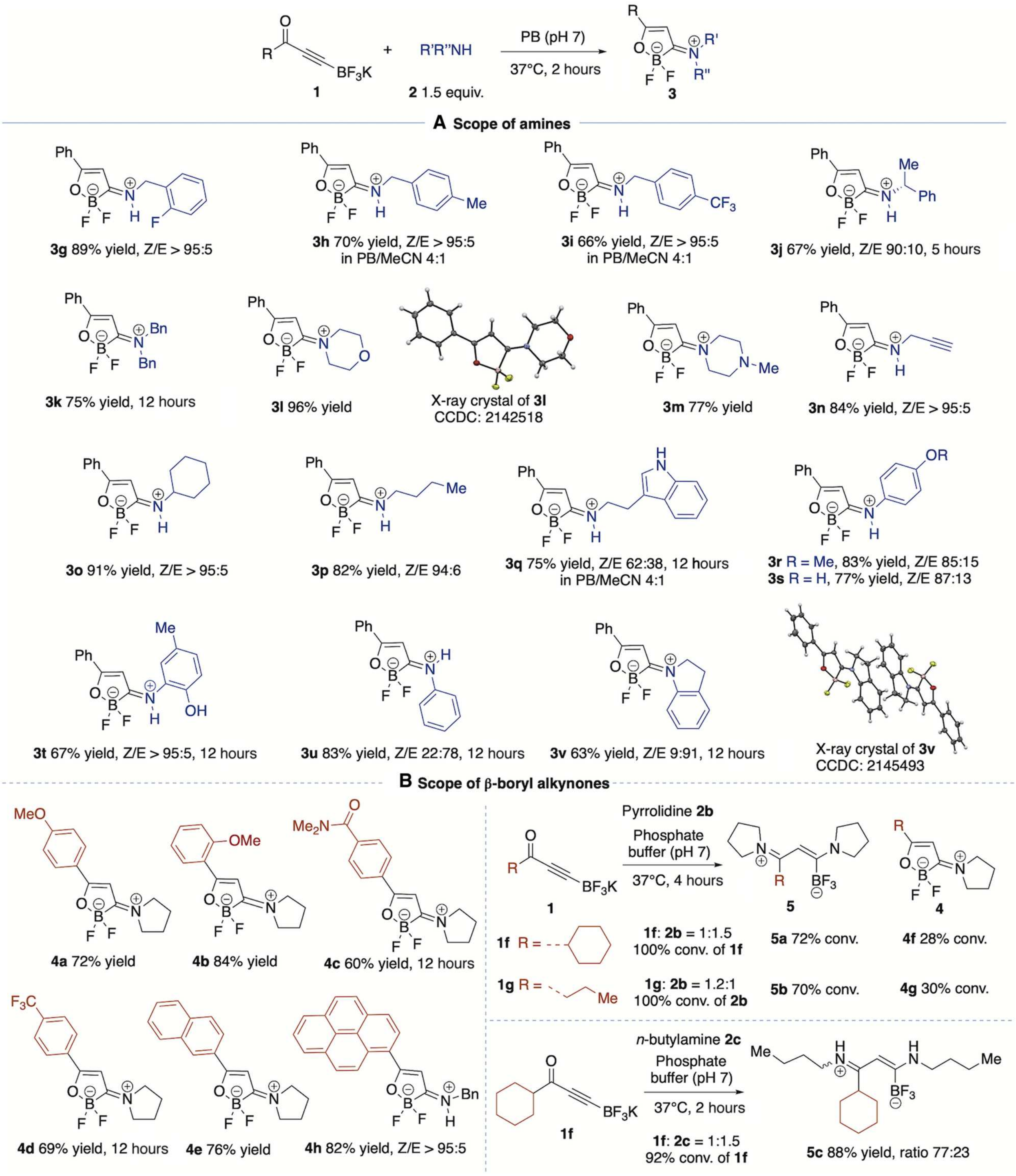


Fig. 3. Substrate scope in the reaction of amine nucleophiles to alkynone β-trifluoroborates. (**A**) Scope of amines. (**B**) Scope of β-boryl alkynones.

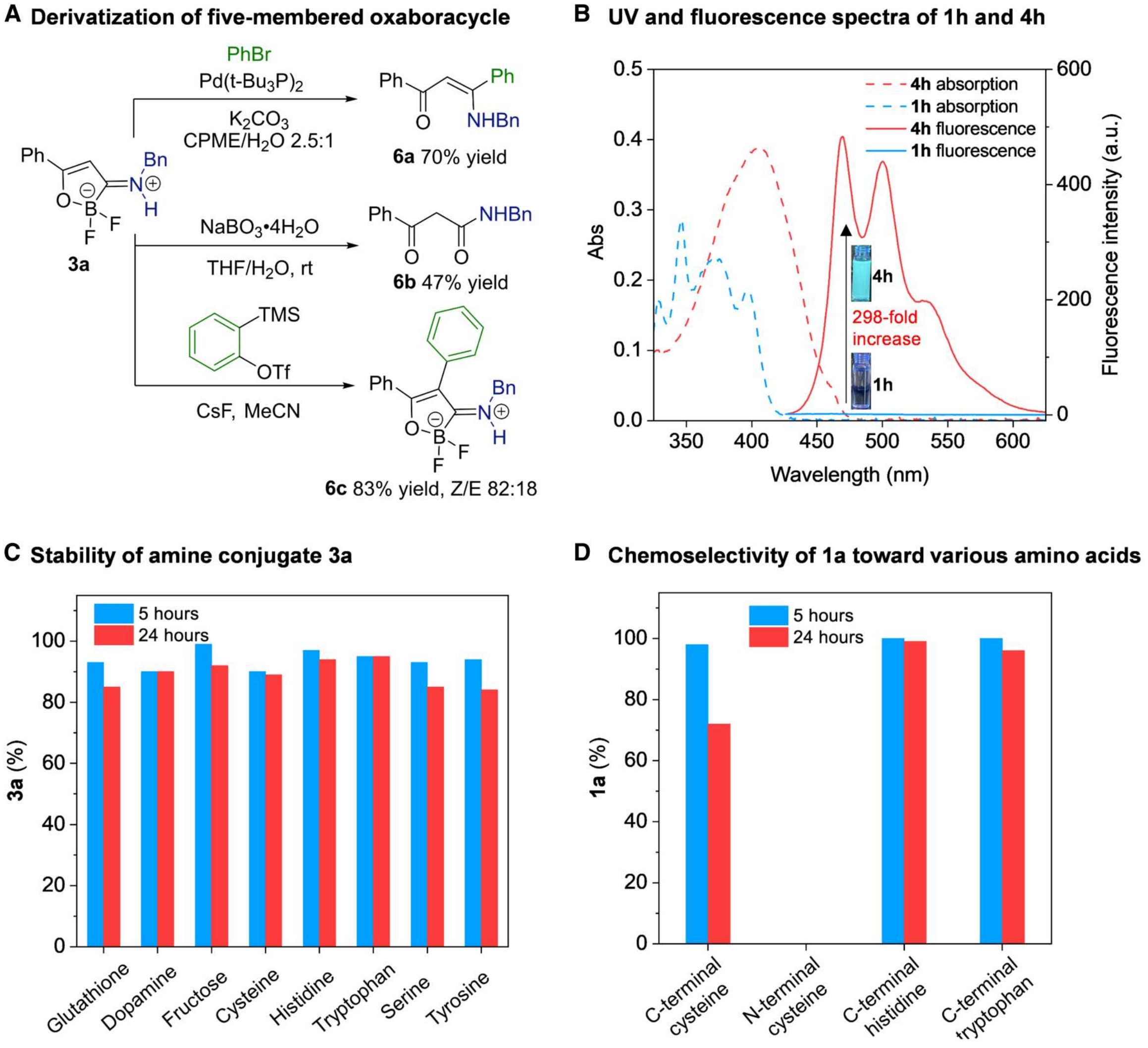


Fig. 4. Versatility of conjugate adducts and applicability of this methodology to bioconjugation. (**A**) Derivatization of the conjugated product. (**B**) Absorption and emission spectra of **1h** and **4h** (12 μM in THF, λ_{exc} = 406 nm). UV, ultraviolet. (**C**) Stability of amine conjugate **3a**. (**D**) Chemoselectivity of **1a** toward various amino acids. a.u., arbitrary units.

that **4h** containing the oxaboracycle structure was much more fluorogenic than **1h**, exhibiting a 298-fold increased fluorescent emission at about 470 nm under the excitation of 406 nm light. Therefore, alkynone β-trifluoroborates such as **1h** could serve as a fluorescent light-up probe upon reaction with amine groups (*60*, *61*).

To further demonstrate the potential of this methodology in bioconjugation, we studied the reaction kinetics using ^{1}H nuclear magnetic resonance (NMR) and ultra performance liquid chromatography. The half-life of β-boryl alkynone reagent **1a** (0.25 M) in the reaction of benzylamine was determined to be 12 min (fig. S4), and the observed second-order rate constant was calculated to be 0.23 M^{-1} min^{-1} (fig. S6), which were comparable to the previous values for the generation of five-membered boracycles from nonactivated amine reactants (*35*, *62*). Next, we investigated the stability of the oxaboracycle product. In the presence of various amino acids and other endogenous molecules, no significant changes in the percentage of the amine conjugate **3a** were detected (Fig. 4C). In contrast, iminoboronates were prone to hydrolysis by the addition of glutathione, dopamine, and fructose (*28*). Product **3a** was stable in pH-neutral buffer over 1 week and exhibited high resistance to degradation under acidic (pH 2), basic (pH 10), and oxidative (10 mM H_2O_2) conditions (figs. S8 and S9). We also examined the chemoselectivity of β-boryl alkynone reagent **1a**. Incubation of **1a** with nucleophilic C-terminal amino acids such as cysteine, histidine, and tryptophan resulted in no reaction (Fig. 4D). Notably, **1a** could react with N-terminal cysteine in ammonium bicarbonate buffer to release the exclusive amine addition product **4i** (Fig. 5A). This intramolecular competition experiment displayed high specificity of alkynone β-trifluoroborates to amines in the presence of thiols. Together, the comparable reaction rate, remarkable product stability, and excellent chemoselectivity support the high potential of this chemical approach in the amine-specific bioconjugation.

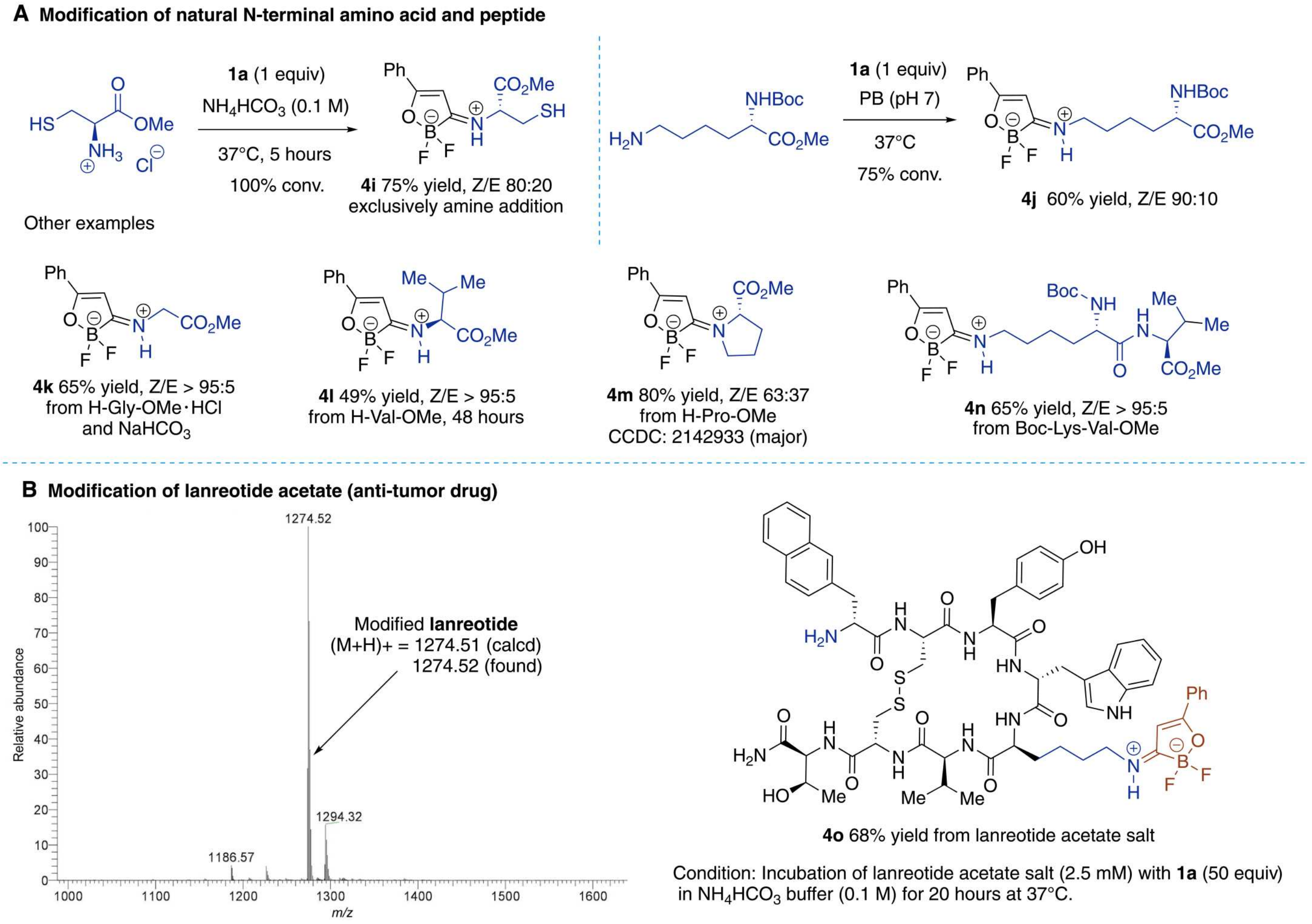


Fig. 5. Amine conjugation of the boryl reagent with N-terminal amino acids and peptides. (**A**) Modification of N-terminal amino acids and peptides using **1a**. (**B**) Modification of antitumor lanreotide acetate using **1a**.

Modification on amino acids, peptide, and proteins

In the evaluation of amine-specific conjugation in biomolecules, a series of N-terminal amino acid esters were first incubated with the boron reagent under physiological conditions (Fig. 5A). For instance, 1-phenylprop-2-ynone-3-trifluoroborate **1a** was readily added to the ε-amino residue of the *N*-Boc-lysine and glycine derivatives. Similar to the result for pyrrolidine, *L*-proline methyl ester could also be effectively converted to the oxaboracycle, which was confirmed by x-ray crystallography analysis. The reaction of branched amino acid derivatives such as valine methyl ester, which had a bulky α-isopropyl group, delivered **4l** in a prolonged reaction time. Encouraged by these promising outcomes, we further investigated the robustness of this methodology in peptides. When the synthesized Lys-Val-OMe was treated with **1a** in phosphate buffer at 37°C, the lysine residue in the dipeptide sequence could be successfully functionalized to provide the conjugated adduct **4n**. Next, commercialized oxytocin, which has only one branched amino acid residue, was incubated with **1a** in NH_4OAc buffer. However, no modification product was detected in liquid chromatography–mass spectrometry (LC-MS) spectra. Another cyclic peptide, lanreotide acetate salt, which has one lysine residue and one branched α-amino group, was mixed with **1a** in NH_4HCO_3 buffer. Very gratifyingly, the conjugate was readily obtained with only one modification (Fig. 5B). On the basis of the result of oxytocin, it was most likely that **1a** selectively added to the linear amino group from lysine, while the branched amino acid residue remained intact.

Taking these results into consideration, we next attempted the modification of more complex biomolecules, i.e., proteins (Fig. 6). Lysozyme is a protein from the hen egg white containing six lysine residues and one N terminus amine. Incubation of lysozyme with 15 equivalents of β-boryl alkynone reagent **1a** in an ammonium acetate buffer (20 mM) generated the boron-containing conjugates at the maximum of sevenfold modifications, which were identified in electrospray ionization (ESI)–MS analysis. Similarly, we were glad to observe that alkynone trifluoroborate **1a** enabled full conversion of ribonuclease A in a short reaction time. The labeling of ribonuclease A can also be achieved by pyrene-attached alkynone reagent **1h**, providing the conjugated species with multiple functionalities (*63*). Noticeable fluorescence in the SDS–polyacrylamide gel electrophoresis (SDS-PAGE) analysis for **4r** provided an additional validation of the protein labeling reaction (fig. S20B). In contrast,

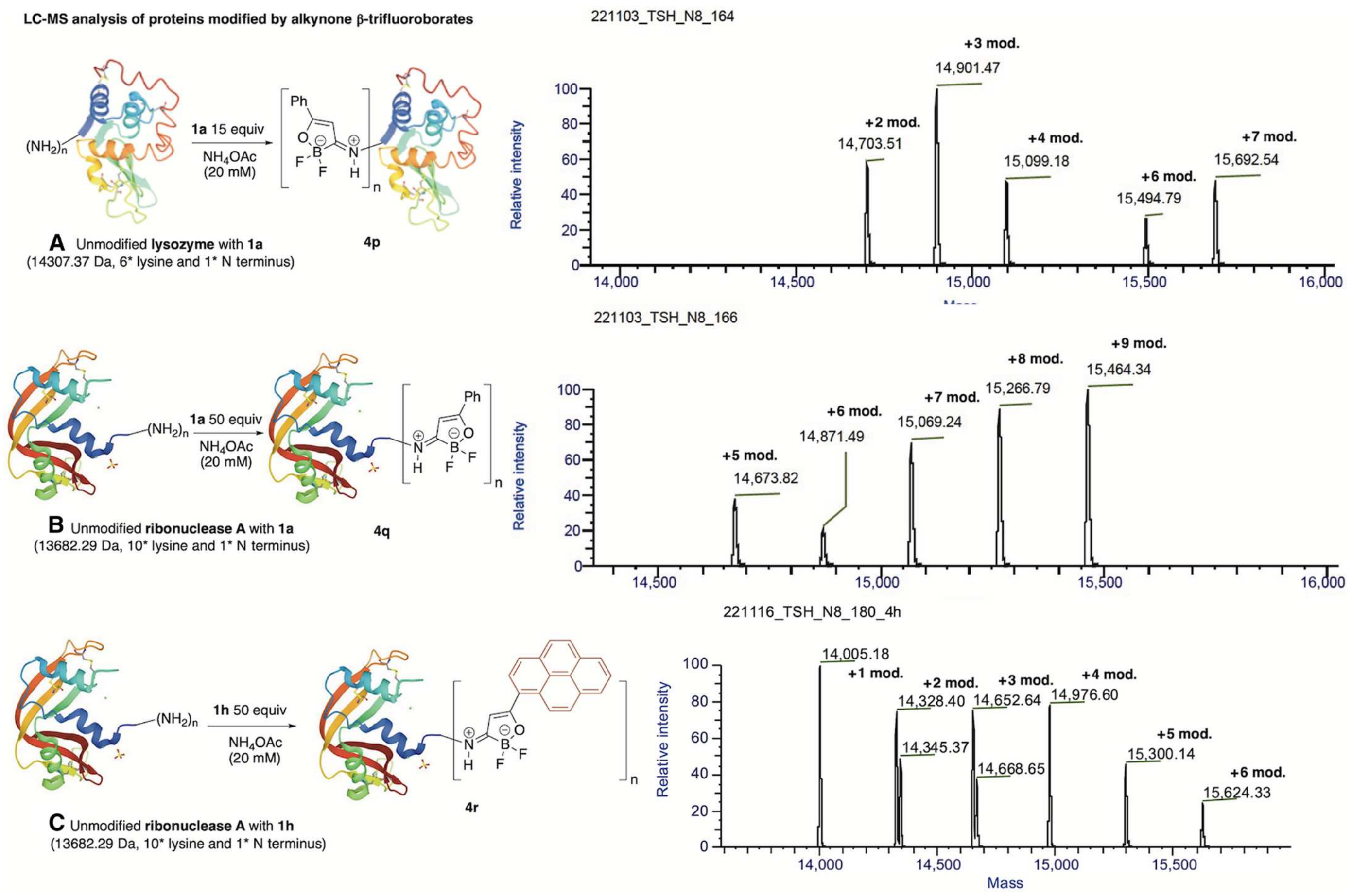


Fig. 6. LC-MS analysis of proteins modified by alkynone β-trifluoroborates. Reaction of lysozyme and ribonuclease A with **1** in NH_4OAc (20 mM) at 37°C for 2 to 4 hours.

unmodified ribonuclease A was not fluorogenic in protein gels. Thus, our currently developed conjugation method can be applied to the development of therapeutic antibodies (*64*), where multiple conjugations are desired.

DISCUSSION

To shed light on the details of the reaction pathway and the essential role of water, the amine conjugation was performed in deuterium oxide at 37°C within 2 hours (Fig. 7A). As expected, a clean deuterium labeling on the vinyl carbon (96% D) took place in the reaction between benzylamine and **1a**. For the conjugation of a secondary amine to **1e**, 93% of the deuterium rate was observed at the enamine carbon by ^1H NMR spectroscopy analysis. Both results suggested the involvement of water in the protonation step, as the proton of vinyl carbon in product **3a** and **3e** came from the water rather than the amine itself. Furthermore, DFT calculations (Fig. 7B) showed that water acts as a proton shuttle and thereby lowers the barrier for this otherwise infeasible proton transfer step after 1,4-conjugate addition (by ~13 kcal/mol, compare **8*** and **8*_H$_2$O**), highlighting the key role of water in this reaction, as observed experimentally (the catalytic water molecule is already highly solvated by other water molecules before the reaction, whereas our model adopts the ideal gas approximation. Therefore, the calculated free energy increase when forming the water prereaction complex **7_H2O** from **7** is probably overestimated). Last, a plausible mechanism is depicted in Fig. 7C. In the case of aromatic alkynones, 1,4-conjugate addition of **1a** took place, followed by the water-assisted protonation, generating the labile enaminone β-trifluoroborate in two resonance forms, **Int-1** and **Int-2**. The latter having a planar iminium structure was more likely to form owing to the conjugation stabilization. Promoted by the coordination of carbonyl oxygen atom to the Lewis acidic boron center, β-trifluoroborate underwent the intramolecular ligand exchange, furnishing the predominant bench-stable five-membered oxaboracycle 3. In terms of the reactions with aliphatic alkynones and alkynal, 1,2-addition of a second amine molecule onto the enaminone intermediate could occur as the competing pathway, affording the iminium trifluoroborates 5 as the major product (*65*). Given the possibility of the ionic attraction with potassium cation, the alternative sequence involving the iminium formation (1,2-addition) before Michael addition (1,4-conjugate addition) cannot be excluded.

In summary, we have found that alkynone β-trifluoroborate is a new class of amine-specific bioconjugation reagent. Compared to the other acetylene Michael acceptors, the incorporation of the

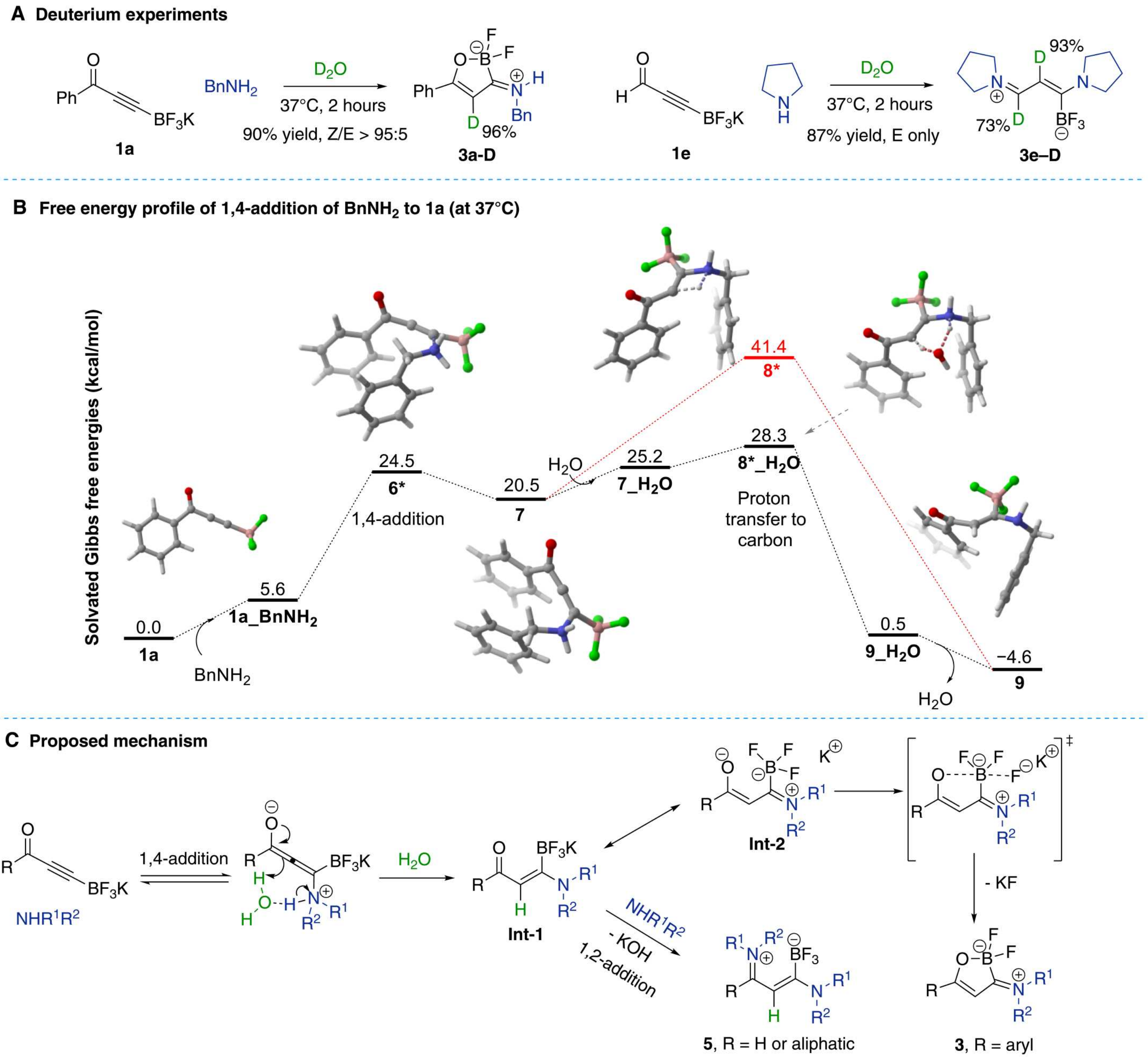


Fig. 7. Mechanistic studies. (**A**) Deuterium experiments. (**B**) Free energy profile of 1,4-addition of BnNH$_2$ to **1a** (at 37°C), determined at the M06-2X/def2-TZVP(water-SMD) //M06-2X/6-31G*(water-SMD) level. (**C**) Mechanistic proposal.

additional boron functionality notably improved the reactivity preferentially to amines over thiols and suppressed the retro process via the dative B─O bond formation. The resulting products are stable in the presence of natural amino acids and polyols. No degradation of these amine conjugates was observed under the reducing (glutathione), oxidative (H_2O_2), acidic, or basic conditions. The oxaboracycle compounds displayed unique synthetic utility and provided a novel fluorophore motif. This chemical approach satisfies the criteria of click reaction including benign reaction conditions, operational simplicity, high atom economy, remarkable product stability, and exquisite chemoselectivity. Therefore, the conjugate addition of simple amines can be extended to biomolecules including natural amino acid esters, peptides, and even more complex proteins. On the basis of the mechanistic studies, plausible reaction pathways were presented, which elucidated the key role of water. The high specificity of alkynone β-trifluoroborates toward amines over thiols is remarkable and should be applicable to the design of lysine-targeting covalent inhibitors. We envision that this conjugation technology will also bring inspiration to researchers who work on antibody-drug conjugates.

MATERIALS AND METHODS

General procedure for amine addition to alkynone β-trifluoroborates

In a 4-ml reaction tube, pH-neutral phosphate buffer (4 ml/mmol of alkynone β-trifluoroborate) was added to the mixture of **1** (1 equiv) and amine nucleophile **2** (1.5 equiv). After stirring vigorously for 2 hours at 37°C, the emulsion was directly evaporated under the reduced pressure. The crude product was then subjected to ^{1}H NMR to determine the Z/E ratio. Unless otherwise noted, Z and E isomers can be separated by silica. For ^{13}C NMR, the carbon directly attached to the boron atom was not detected, likely due to quadrupolar relaxation. In the absence of x-ray crystallography data, the assignment of Z and E configuration was based on the comparison of chemical shifts in ^{19}F NMR.

General procedure for stability studies of the conjugate product

In a 4-ml reaction tube, the endogenous molecule (1 equiv) was added to **3a** (5.7 mg, 0.02 mmol) in aqueous buffer solution (0.4 ml). After stirring for the indicated time (5 and 24 hours) at 37°C, the reaction was removed from the stir plate and concentrated under the reduced pressure. To the same tube, dibromomethane (7 μl, 0.1 mmol) was added as the internal standard and dissolved in CD_3CN. Subsequently, a ^{1}H NMR was collected to evaluate the stability of the conjugate product.

Supplementary Materials

This PDF file includes:

Figs. S1 to S31
Tables S1 to S3
References

View/request a protocol for this paper from *Bio-protocol*.

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