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Affinity-Labeling-Based Introduction of a Reactive Handle for Natural Protein Modification

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Abstract: A new chemical method to site-specifically modify natural proteins without the need for genetic manipulation is described. Our strategy involves the affinity-labeling-based attachment of a unique reactive handle at the surface of the target protein, and the subsequent selective transformation of the reactive handle by a bioorthogonal reaction to introduce a variety of functional probes into the protein. To demonstrate this approach, we synthesized labeling reagents that contain: 1) a benzenesulfonamide ligand that directs specifically to bovine carbonic anhydrase II (bCA), 2) an electrophilic epoxide group for protein labeling, 3) an exchangeable hydrazone bond linking the ligand and the epoxide group, and 4) an iodophenyl or acetylene handle. By incubating the labeling reagent with bCA, the reactive handle was covalently attached at the surface of bCA through epoxide ring opening. Either after or before removing the ligand by a hydrazone/oxime-exhange reaction, which restores the enzymatic activity, the reactive handle incorporated could be derivatized by Suzuki coupling or Huisgen cycloaddition reactions. This method is also applicable to the target-specific multiple modification in a protein mixture. The availability of various (photo)affinity-labeling reagents and bioorthogonal reactions should extend the flexibility of this strategy for the site-selective incorporation of many functional molecules into proteins.

Keywords: affinity labeling · bioorthogonal reactions · protein engineering · protein modifications · target-specific labeling

Introduction

Incorporation of functional molecules other than naturally encoded amino acids, such as fluorescent probes, affinity tags, artificial receptors, and synthetic polymers, into proteins represents a powerful approach for protein engineering.[1] Consequently, there is an ever-increasing need for new protein-modification methods. While significant advances have been made in molecular-biology-based techniques for unnatural amino acid mutagenesis,[2] there is considerable interest in exploring and developing new chemistry-based methods that can be used for the specific modification of premade proteins under mild aqueous conditions.[3] To complement traditional bioconjugation reactions that most commonly target lysine or cysteine residues in proteins,[4] several new entries such as transition metal-catalyzed reactions[5] and a Mannich-type reaction[6] have been recently developed that can functionalize rarely occurring aromatic amino acids that are exposed on the surface of proteins. Although these sophisticated reactions expand the flexibility of protein bioconjugation, they are only residue-specific, and thus the modification sites and numbers cannot be sufficiently controlled. Therefore, selective chemical reactions that are orthogonal to the functionalities present in natural proteins have been designed as an alternative strategy. Recently, the Staudinger ligation[7] and the Huisgen [3+2] cycloaddition reaction (click chemistry)[8] have proven to be useful for modifying proteins under physiological conditions. However, these methods inevitably require pre-incorporation of an azide or an acetylene moiety as a reactive handle within the framework of the target protein by genetic engineering. More simple and straightforward chemical methods without genetic operation should be desired. Herein, we describe a new chemical strategy for introducing unique reactive handles into natural proteins without the need for genetic manipulation. This method enables the active-site-directed at-

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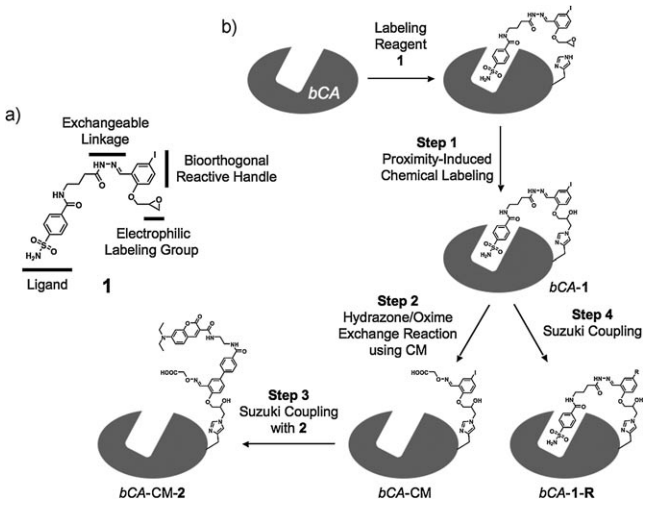
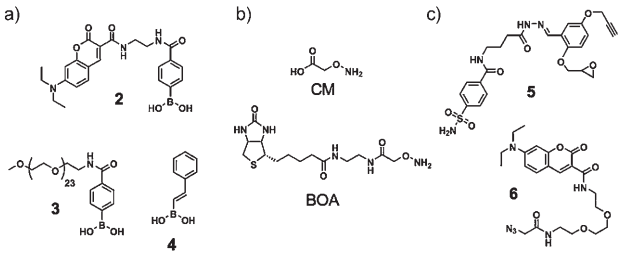
tachment of a reactive handle to natural protein surfaces, which is valuable for applying a variety of bioorthogonal reactions[9] as tools to the manipulation of protein structure and function.

Results and Discussion

Our strategy is depicted in Scheme 1. For introducing a reactive handle to the surface of natural proteins, we applied the post-affinity-labeling modification (P-ALM) method that was recently developed by our group.[10] A key advantage of this strategy is that the ligand-directed labeling occurs specifically in the vicinity of ligand-binding (catalytic) pocket of the target protein. As a bioorthogonal chemical reaction, Suzuki coupling,[11] a representative organometallic reaction that creates a carbon–carbon bond, and the well-established Huisgen reaction[8] were employed in this study. In particular, the applicability of palladium-catalyzed cross-coupling reactions for protein and peptide modification has been recently demonstrated by our[12] and other[13] groups. As a model enzyme, we chose bovine carbonic anhydrase II (bCA) and thus designed a labeling reagent **1** shown in Scheme 1a. The labeling reagent **1** contains four key functionalities: 1) a benezenesulfonamide ligand that directs specifically to bCA, 2) an electrophilic epoxide group for protein labeling, 3) an exchangeable hydrazone bond that links the ligand and the epoxide group, and 4) an iodophenyl group as a reactive handle for Suzuki coupling. In the case of a Huisgen cycloaddition reaction, we used a labeling reagent **5** that bears an acetylene handle and the corresponding azide-tethered reactant **6** (Scheme 2c). By incubation of the labeling reagent with bCA, the iodophenyl group can be covalently attached at the surface of bCA by the epoxide ring opening. Either after or before removing the ligand by means of a hydrazone/oxime exchange reaction for restoring the enzymatic activity, the iodophenyl handle thus incorporated can be derivatized by a Suzuki coupling reaction with a variety of phenylboronic acid-appended functional molecules.



Scheme 1. Chemical structure of the labeling reagent **1** (a) and schematic illustration of the present strategy (b). See also Scheme 2 for the molecular structure of the reagents.



Scheme 2. Molecules used in this study. a) Phenylboronic acid derivatives for the Suzuki coupling reaction. b) Aminooxy derivatives for hydrazone/oxime-exchange reaction. c) Labeling reagent and its reactant for the protein modification based on the Huisgen reaction.

MALDI-TOF mass analysis revealed that the labeling of native bCA by using **1** was completed after 12 h (step 1), yielding the modified bCA-**1** containing a single iodophenyl group (Figure 1a). No labeling was observed in the presence of the strong inhibitor 6-ethoxy-2-benzothiazolesulfonamide (ET), which indicates that the labeling occurred by an affinity-driven reaction. After purification of bCA-**1** by size-exclusion gel chromatography followed by dialysis, bCA-**1** was subsequently subjected to a hydrazone/oxime-exchange reaction to replace the benzenesulfonamide ligand with carboxymethyloxyamine (CM) to produce bCA-CM (step 2) as previously reported (Figure 1a).[10] We next performed a Suzuki coupling reaction of bCA-CM with a phenylboronic acid derivative of diethylaminocoumarin (DEAC) **2** by using palladium acetate ($Pd(OAc)_2$) as a water-soluble catalyst (step 3). The yield of reaction for the Suzuki coupling varied depending on the reaction conditions including the

Abstract in Japanese:

蛋白質への機能性分子導入法の開発は蛋白質工学における重要な課題の一つである。本論文では、遺伝子操作を必要とせずに天然蛋白質に適用可能な新しい蛋白質修飾法について報告する。本手法では、標的蛋白質に対するリガンドと求電子性エポキシ基をヒドラゾン結合で連結し、更に生体直交性のリアクティブハンドル（ヨードフェニルあるいはアセチレン基）を導入したアフィニティラベル化剤を用いた。このラベル化剤を用いることで、標的蛋白質（炭酸脱水酵素）の部位特異的にリアクティブハンドルを導入することができ、その後、鈴木カップリングもしくはHuisgen環化反応によってこのハンドルに様々な機能性分子を導入できることが実証された。また本手法は、複数の蛋白質混合下における標的蛋白質選択的な二重修飾への応用も可能であった。本コンセプトは、様々なアフィニティラベル化試薬や生体直交性有機反応にも適用できるため、蛋白質修飾のための一般的な手法としての展開が期待される。

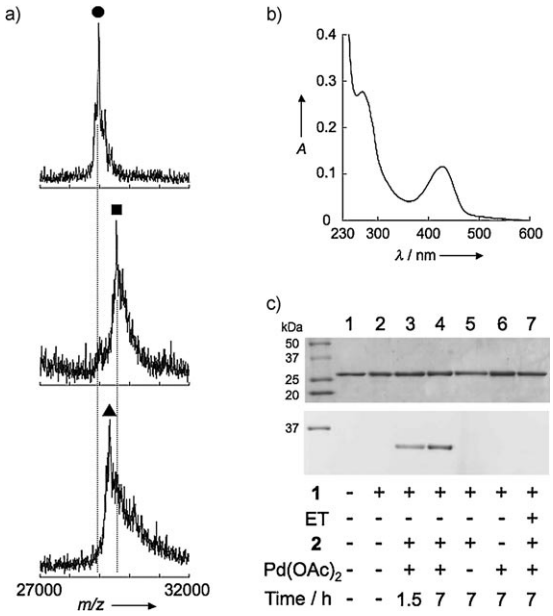


Figure 1. Affinity labeling, hydrazone/oxime-exchange reaction, and Suzuki coupling reaction on the surface of bCA. a) MALDI-TOF MS analyses of bCA before labeling (native bCA, top), after labeling with **1** (bCA-**1**, middle), and after subsequent treatment with CM (bCA-CM, bottom). (●) calcd 28986, obs 28969; (■) calcd 29572, obs 29564; (▲) calcd 29363, obs 29292. Reaction conditions: affinity labeling, 100 μM bCA, 250 μM **1**, 50 mM HEPES buffer solution pH 8.0, 12 h; exchange reaction, 100 μM bCA-**1**, 3 mM CM, 50 mM acetate, pH 5.5, 48 h. b) Absorption spectrum of bCA-CM-**2** after purification. Reaction condition: 1 mM **2**, 1 mM $Pd(OAc)_2$, 10% glycerol, 10% DMSO, 50 mM HEPES buffer solution, pH 8.0, 7 h. c) One-pot affinity labeling and Suzuki coupling reaction of bCA monitored by SDS-PAGE. Top, coomassie brilliant blue (CBB)-staining image; bottom, fluorescence image. Reaction conditions: affinity labeling, 100 μM bCA, 250 μM **1**, 50 mM HEPES buffer solution, pH 8.0, 12 h in the presence or absence of 1 mM ET, then 1 mM **2**, 1 mM $Pd(OAc)_2$, 10% glycerol, 10% DMSO. HEPES = *N*-(2-hydroxyethyl)-piperazine-*N*′-2-ethane sulfonic acid.

concentrations of $Pd(OAc)_2$ and **2**, the types of phosphine ligands, the pH value, the temperature, and the incubation time (Table 1). After the optimization of the reaction conditions, DEAC-coupled bCA-CM-**2** was obtained in approximately 80% yield (Figure 1b). As shown in Figure 1c, in which bCA-**1** was used, in the absence of either the palladium catalyst (lane 5) or phenylboronic acid **2** (lane 6), the DEAC modification did not take place. Also, native bCA, which lacks the iodophenyl group, showed no reactivity toward Suzuki coupling (lane 7). The expected cross-coupling product was further characterized by conventional peptide mapping experiments by using lysyl endopeptidase digestion followed by HPLC and MALDI-TOF MS (see Figure S1 in the Supporting Information). It was revealed that the L1 fragment corresponding to Ser2-Lys9 was selectively modified to be consistent with our previous reports,[10] which suggests the labeling site to be His3 or His4. The enzymatic activity of bCA-**1** was almost completely suppressed because the active site was efficiently masked by the modified benzenesulfonamide ligand. On the other hand, bCA-CM obtained after the ligand-exchange reaction (step 2)

Table 1. Post-affinity-labeling modification of bCA by bioorthogonal reactions.[a]

Entry	bCA	Reactant[b]	Other reagents	Time [h]	Yield [%]
1	bCA-**1**	**2**	1 mM $Pd(OAc)_2$	6	75[c]
2	bCA-**1**	**2**	1 mM $Pd(OAc)_2$	12	80[c]
3	bCA-**1**	**2**	0.5 mM $Pd(OAc)_2$	12	61[c]
4	bCA-**1**	**2**	1 mM $Pd(OAc)_2$, 1 mM TPPTS	12	32[c]
5	bCA-**1**	**2**	1 mM Na_2PdCl_4	12	58[c]
6	bCA-**1**	**2**	1 mM Na_2PdCl_4, 1 mM TPPTS	12	41[c]
7	bCA-CM	**2**	1 mM $Pd(OAc)_2$	7	80
8	bCA-**1**	**3**	1 mM $Pd(OAc)_2$	12	40[d]
9	bCA-**1**	**4**	1 mM $Pd(OAc)_2$	7	–[e]
10	bCA-**5**	**6**	1 mM ascorbic acid, 1 mM $CuSO_4$, and 1 mM tris-triazole ligand	6	87

[a] Conditions: 50 mM HEPES buffer solution, pH 8.0, 10% DMSO, 10% glycerol (entry 1–9), at 37 °C, 50-100 μM modified bCA. [b] 1 mM **2**–**4** or 0.5 mM **6**. [c] Yields were determined by SDS-PAGE/fluorescence imaging by using purified bCA-CM-**2** (entry 7) as a standard sample. [d] Yield was estimated by MALDI-TOF MS. [e] Not determined. TPPTS = triphenylphosphane trisulfonate.

showed a restored activity with a V_{max} value ($15.9 \pm 1.3\,M\,s^{-1}$), which is approximately 33% that of native bCA, and is comparable with that of bCA-CM-**2** ($15.9 \pm 1.2\,M\,s^{-1}$). Therefore, it was concluded that the post-affinity-labeling modification such as the Suzuki coupling reaction did not have detrimental effects on the catalytic activity of bCA.

At step 3 or 4, various functional molecules may also be incorporated into native bCA. For example, a polyethyleneglycol (PEG) was successfully attached to bCA-**1**, so-called PEGylation, by using the phenylboronic acid derivative **3** in moderate yields of approximately 40% (data not shown). Interestingly, a fluorescent stilbene dye could be newly constructed on the bCA surface upon the cross-coupling reaction with styrene boronic acid **4** (Figure 2a). Furthermore,

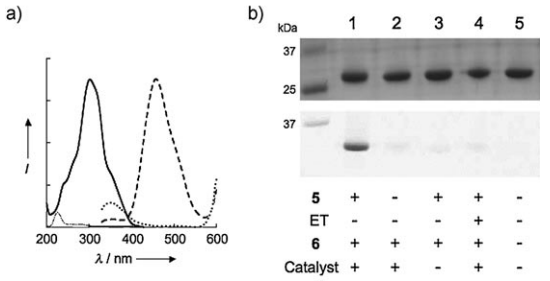


Figure 2. Chemical modification of bCA with various synthetic molecules. a) Stilbene formation on bCA monitored by fluorescence spectroscopy. Excitation spectrum of bCA-**1**-**4** (——); emission spectrum of bCA-**1**-**4** (- - - - -); emission spectrum of bCA-**1** (••••); excitation spectrum of bCA-**1** (–•–•). $\lambda_{em} = 458$ nm, $\lambda_{ex} = 300$ nm. b) One-pot affinity labeling and the Huisgen reaction of bCA monitored by SDS-PAGE. Top, CBB-staining image; bottom, fluorescence image. Reaction conditions: affinity labeling, 100 μM bCA, 250 μM **5**, 50 mM HEPES buffer solution, pH 8.0, in the presence or absence of 1 mM ET, then 500 μM **6**, 1 mM each catalyst[8b,14] (ascorbic acid, $CuSO_4$, and tris-triazole ligand), 10% DMSO.

the present strategy can in principle be generally applicable for using other bioorthogonal chemistry such as the azide–alkyne cycloaddition reaction. When we used the labeling reagent **5** and DEAC **6** (Scheme 2c), it was confirmed that **5** reacts with bCA in a quantitative yield and the acetylene-tagged bCA obtained was modified with **6** in the presence of copper(I) ions and organic tetrazole catalysts (see Figure S2 in the Supporting Information)[8b,14] to afford the fluorescently labeled bCA-**5**-**6** in 87% yield. All these results clearly demonstrated that the general strategy proposed in Scheme 1 was carried out as a proof of principle.

By taking advantage of the selectivity of this strategy imposed by the affinity labeling, Suzuki coupling, and the hydrazone/oxime-exchange reaction, all of which are orthogonal to each other, it was possible to perform dual labeling of bCA (Figure 3). After the affinity labeling by using **1** and the subsequent Suzuki coupling with **2** to yield bCA-**1**-**2**, the hydrazone/oxime-exchange reaction was carried out by treatment with oxyamine-appended biotin (BOA). As shown in Figure 3a, only bCA-BOA-**2** containing the DEAC fluorophore and biotin tag, but not bCA-**1**-**2**, was efficiently captured on an avidin-immobilized resin and eluted with biotin. In addition, this procedure was successfully applied for the selective dual labeling of bCA in a protein mixture (Figure 3b). Incubation of an equimolar mixture of four proteins (β-galactosidase (β-Gal), bovine serum albumin (BSA), bCA, glutathione *S*-transferase (GST), and hemoglobin (Hb)) in the presence of labeling reagent **1** and an in situ Suzuki coupling reaction with **2** resulted in highly selective labeling of bCA (lane 2 of fluorescence image). Subsequent addition of BOA to the above mixture resulted in the replacement of the sulfonamide ligand with biotin to yield the bCA-BOA-**2** (lane 2 of DAB staining image). Nonspecific labeling to other proteins was never observed in the presence of ET (lane 3). It should be noted that all reaction steps were carried out in one pot without any purification step. These results indicate that target-specific multiple labeling is feasible even in protein mixtures by coupling the P-ALM method[10] and bioorthogonal chemistry.

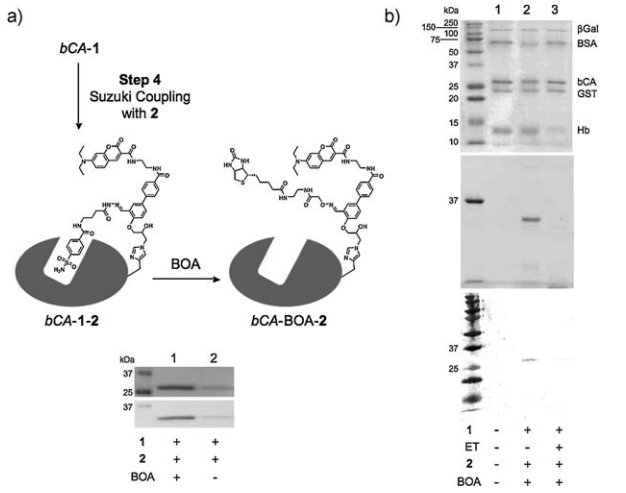


Figure 3. Dual labeling of bCA in situ. a) Isolation of dual-labeled bCA-BOA-**2** by an avidin-immobilized column. After preparing bCA-**1**-**2**, the hydrazone/oxime-exchange reaction was performed with BOA. The bCA-BOA-**2** obtained was captured on a SoftLink Soft Release Avidin Resin, washed, and eluted with biotin. The eluted fraction was applied to SDS-PAGE. Top, CBB-staining image; bottom, fluorescence image. Reaction conditions: affinity labeling and Suzuki coupling conditions (identical with the conditions listed for Figure 1C); exchange reaction, 3 mM BOA, 50 mM Acetate pH 5.5. b) Dual labeling in a protein mixture. Top, CBB-staining image; middle, fluorescence image; bottom, diaminobenzidine (DAB)-staining image. Reaction conditions: 1) affinity labeling, 150 μg mL^{-1} each protein (5 μM bCA), 12.5 μM **1**, 50 mM HEPES buffer solution, pH 8.0, in the presence or absence of 100 μM ET; 2) Suzuki coupling, 1 mM **2**, 1 mM $Pd(OAc)_2$, 10% glycerol, 10% DMSO; 3) hydrazone/oxime exchange reaction, 3 mM BOA.

Conclusions

We demonstrated that the affinity-labeling-based introduction of unique reactive handles into proteins is valuable as a new method for chemically modifying natural proteins. The availability of various (photo)affinity-labeling reagents[15] and bioorthogonal reactions[9] should extend the flexibility of this strategy for the site-selective incorporation of many functional molecules into proteins, which will provide powerful tools for protein engineering. We are currently working on this line of work.

Experimental Section

Synthesis

Detailed synthetic procedures and compound characterizations are described in the Supporting Information.

General Methods for Biochemical Assays

MALDI-TOF MS was recorded on PE Voyager DE-RP (Applied Biosystems). Sinapic acid (SA) or α-cyano-4-hydroxycinnamic acid (CHCA) were used as a matrix. Reversed-phase HPLC (RP-HPLC) was carried out on an ODS column (YMC-Pack ODS-A) by using a Hitachi L-7100 HPLC system. UV/Vis spectra were recorded on a Shimadzu UV-visible 2550 spectrometer. Fluorescence spectra were recorded on a Perkin–Elmer LS55 spectrometer. Fluorescence gel images were acquired by using a ChemiDoc XRS (BioRad) with a 480BP70 bandpass filter.

The bovine carbonic anhydrase II (bCA, Sigma) solution was prepared in 50 mM HEPES buffer solution (pH 8.0) and the concentration was determined by measuring the absorbance at 280 nm ($\varepsilon_{280} = 53{,}070\ M^{-1}cm^{-1}$).[16]

Affinity labeling, ligand exchange, and the Suzuki coupling reaction on bCA to yield bCA-CM-2 (Step 1→2→3, Figure 1a and b)

The affinity-labeling reaction was performed under the following condition at 37°C for 12 h to obtain bCA-**1**: 100 μM bCA, 250 μM **1**, 50 mM HEPES buffer solution (pH 8.0) containing 1% dimethyl sulfoxide (DMSO). After the reaction solution was dialyzed against 50 mM acetate buffer solution (pH 5.5), the ligand-exchange reaction was initiated by adding CM (200 equiv to bCA) to the solution. After subsequent incubation at 37°C for 48 h to yield bCA-CM, the solution was again dialyzed against 50 mM HEPES buffer solution (pH 8.0). The Suzuki coupling reaction was carried out by adding the following reagents to the above solution and incubating the mixture at 37°C for 7 h: 1 mM **2**, 1 mM $Pd(OAc)_2$, 10% glycerol, 10% DMSO. The bCA-CM-**2** thus obtained was purified by treating the solution with ethylenediamine (50 mM) at 37°C for 15 h, which was followed by gel filtration chromatography (TOYOPEARL HW-40F, TOSOH Corp.; eluent: 50 mM HEPES buffer solution

(pH 8.0)). It should be noted that the ethylenediamine treatment was essential to remove the palladium ion efficiently.

The affinity-labeling reaction and ligand-exchange reaction were monitored by MALDI-TOF MS. The yield of the Suzuki coupling reaction was determined from the absorbance of bCA-CM-**2** at 280 and 430 nm.

Peptide Mapping Experiments

The bCA-CM-**2** solution (10 μM) was diluted with 100 mM ammonium carbonate (pH 8.5) containing 2 M urea. Lysyl endopeptidase (LEP, Wako) was added so that the LEP/substrate ratio was 1:50 (w/w), and the digestion was allowed to proceed at 37 °C overnight. The digested peptides were separated by RP-HPLC with a linear gradient of acetonitrile containing 0.1 % TFA (solution A) and 0.1 % aqueous TFA (solution B). RP-HPLC was monitored by UV absorbance at 220 nm and fluorescence at 470 nm (λ_{ex} = 430 nm). Each fraction was analyzed by MALDI-TOF MS. HPLC traces of LEP-digested bCAs are shown in Figure S1 in the Supporting Information.

Enzymatic-Activity Assay[10]

The hydrolytic activity of bCAs was assayed in 50 mM HEPES buffer solution (pH 7.2) by using *p*-nitrophenyl acetate as a substrate. Modified bCAs were dialyzed against the same buffer solution before each assay. Reaction conditions were as follows: 1 μM bCA, 0.2–20 mM *p*-nitrophenyl acetate. Initial rates of *p*-nitrophenyl acetate hydrolysis were determined by measuring the increase of absorbance at 348 nm ($\Delta\varepsilon_{348}$ = 5150 $M^{-1}cm^{-1}$)[17] for the initial 30 sec. Kinetic parameters were obtained by fitting a plot of the initial rates as a function of substrate concentration to the Michaelis–Menten Equation (Equation (1)) by using KaleidaGraph (Synergy Software).

$$V_0 = k_{cat} \cdot [E]_0 \cdot [S]/([S] + K_m) \quad (1)$$

V_0 is the initial rate of hydrolysis, k_{cat} and K_m are the first-order rate constant from the catalyst–substrate complex and the Michaelis–Menten constant, respectively. $[E]_0$ and [S] are the initial concentrations of bCA and substrate, respectively.

Affinity Labeling and Suzuki Coupling Reaction on bCA to Yield bCA-1-R (Step 1→4, Figure 1c and Figure 2a)

Affinity-labeling reactions were performed as described above (100 μM bCA, 250 μM **1**, 50 mM HEPES buffer (pH 8.0)). For inhibition experiments, ET (1 mM) was included in the solution. The reaction solutions were directly used for Suzuki coupling reaction by adding the following reagents: 1 mM phenylboronic acid derivatives **2**, **3**, or **4**, 1 mM $Pd(OAc)_2$, 10 % glycerol, 10 % DMSO. For bCA-**1**-**2**, the reaction solution was incubated at 37 °C for 7 h and then analyzed by SDS-PAGE/fluorescence gel imaging. For bCA-**1**-**3**, the reaction solution was incubated at 37 °C for 12 h and then analyzed by MALDI-TOF MS. For bCA-**1**-**4**, the reaction solution was incubated at 37 °C for 7 h, purified by gel filtration chromatography (TOYOPEARL HW-40F; eluent: 50 mM HEPES buffer solution (pH 8.0)), and then fluorescence and excitation spectra were measured.

Dual Labeling and Pull-Down of bCA (Figure 3a)

Affinity labeling and the Suzuki coupling reaction were performed in one pot as described above to yield bCA-**1**-**2**. The reaction solution was dialyzed against 50 mM acetate buffer solution (pH 5.5), and then the ligand-exchange reaction was initiated by adding BOA (3 mM) to the solution. After incubating for 45 h at 37 °C, the solution was dialyzed against 50 mM HEPES buffer solution (pH 8.0) to remove excess BOA. The biotinylated bCA, bCA-**1–2**, was captured on a SoftLink Soft Release Avidin Resin (Promega), washed, and eluted with a biotin solution (25 mM) according to the manufacture's protocol. The eluted fraction was analyzed by SDS-PAGE/fluorescence gel imaging.

Dual Labeling of bCA in a Protein Mixture (Figure 3b)

Affinity labeling in a protein mixture was performed under the following conditions: 150 $\mu g\,mL^{-1}$ each protein (5 μM bCA), 12.5 μM **1**, 50 mM HEPES buffer solution (pH 8.0) containing 1 % DMSO, in the presence or absence of 100 μM ET. Suzuki coupling was carried out as described above by using **2**. Subsequently, the ligand-exchange reaction was initiated by adding BOA (3 mM) to the solution, and the reaction was allowed to proceed for 15 h at 37 °C. The protein samples were fractionated by 12.5 % SDS-PAGE, analyzed by fluorescence gel imaging, and electrotransfered onto a PVDF membrane. The biotinylated bCA, bCA-**1**-**2**, was detected with avidin-peroxidase (Sigma) by using a diaminobenzidine (DAB, Wako).

Affinity Labeling and the Huisgen Cycloaddition Reaction on bCA (Figure 2b)

The affinity-labeling reaction was performed by using **5** under the following condition at 37 °C for 12 h to obtain bCA-**5**: 100 μM bCA, 250 μM **5**, 50 mM HEPES buffer solution (pH 8.0) containing 1 % DMSO, in the presence or absence of 1 mM ET. The reaction solutions were directly used for the Huisgen cycloaddition reaction by adding the following reagents: 500 μM **6**, 1 mM ascorbic acid, 1 mM $CuSO_4$, 1 mM tris-triazole ligand (Figure S2 in the Supporting Information), 10 % DMSO. After incubation at 37 °C for 6 h, the samples were analyzed by SDS-PAGE/fluorescence gel imaging.

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