DOI: 10.1002/ange.201303831

Protein Modification

Ligand-Directed Selective Protein Modification Based on Local Single-Electron-Transfer Catalysis**

*Shinichi Sato and Hiroyuki Nakamura**

Techniques for the visualization of target proteins in living systems are highly important to investigate the function, dynamics, localization, and crosstalk of individual proteins.[1] For this purpose, GFP fusion tags and monoclonal antibodies[2] are widely used for conventional protein labeling in molecular biology. Although these are useful methods for the real-time monitoring of target proteins in living systems, genetic manipulation is often necessary, and the conjugation of these proteins with relatively large labeling groups sometimes results in improper biophysical functions owing to undesirable interactions with other molecules.

The chemical modification of proteins with small-molecule probes has received much interest as an alternative powerful method for the study of individual proteins in their native environments. The key to this chemical modification is a bioorthogonal chemical reaction that enables the rapid and selective bioconjugation of proteins with nonnatural functional groups under physiological conditions. The most widely utilized bioorthogonal chemical reactions rely on electrophilic reagents that target nucleophilic amino acids, such as lysine and cysteine. In addition, the lysine-specific reductive alkylation using an iridium catalyst,[3] the conversion of cysteine into dehydroalanine,[4] and the radical addition reaction of cysteine with an alkene[5] were recently developed. Furthermore, remarkable attention has been paid to the modification of aromatic amino acids, such as tyrosine and tryptophan, by transition-metal-mediated processes,[6–8] the three-component Mannich reaction,[9] the click-like ene-type reaction,[10] the oxidative modification using cerium(IV) ammonium nitrate,[11] and the use of formylbenzene diazonium reagents.[12]

Although various bioorthogonal chemical reactions have been reported, there are still few reports regarding selective chemical modifications of native target proteins. Popp and Ball recently reported the site-specific protein modification of aromatic side chains with dirhodium metallopeptide catalysts.[13,14] Hamachi and co-workers pioneered a ligand-directed protein labeling method in which an electrophilic phenylsulfonate ester[15] or imidazole[16] group conjugated with ligand molecules specifically reacted with the nucleophilic amino acid side chains on the surfaces of target proteins through an S_N2-type reaction with the concomitant release of the ligand molecules. Furthermore, they were the first to demonstrate the catalytic target-selective modification using ligand-tethered *N*,*N*-dimethylaminopyridine (DMAP) catalysts (Figure 1 A), which allowed highly efficient transfer of acyl donor probes to the nucleophilic regions of the targeted protein through an S_N2-type reaction.[17,18] Herein, we report the development of a ligand-directed selective protein modification method based on local single-electron transfer catalysis (Figure 1 B). In this method, a single-electron transfer (SET) with a photocatalyst,[19,20] such as the ruthenium tris(2,2′-bipyridyl) complex ($[Ru(bpy)_3]^{2+}$), is essential for the generation of tyrosyl radicals[21,22] that react with tyrosyl radical trapping agents (TRTs) containing an *N*′-acyl-*N*,*N*-dimethyl-1,4-phenylenediamine through a catalytic oxidative radical addition reaction.

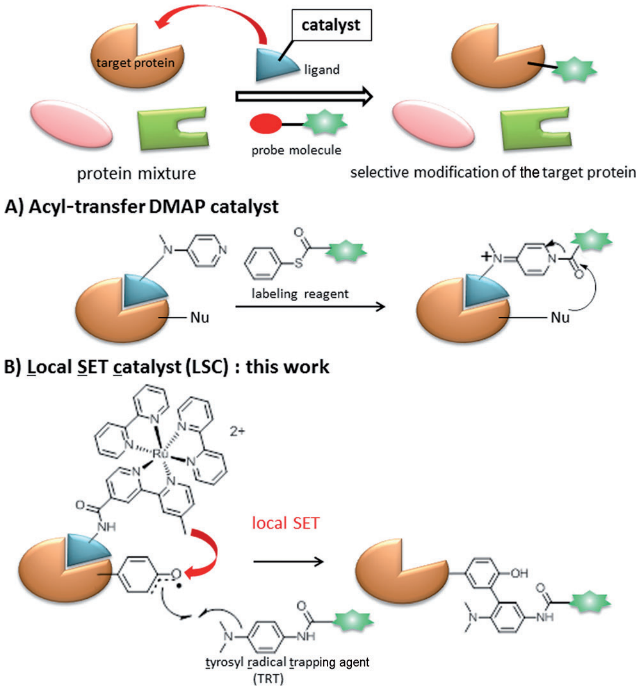


Figure 1. Ligand-directed selective protein modification. A) Acyl-transfer DMAP catalyst promotes the acylation of the nucleophilic regions of the target protein through an S_N2-type reaction. B) Local SET catalyst (LSC; this work) promotes the generation of tyrosyl radicals that react with tyrosyl radical trapping agents (TRTs) in the local environment of the probe on the target protein in the presence of visible light.

---

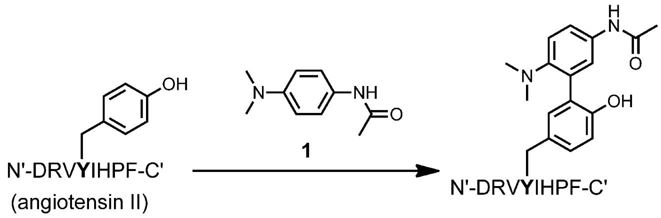
[*] Dr. S. Sato, Prof. H. Nakamura
Department of Chemistry, Faculty of Science, Gakushuin University
Mejiro, Tokyo 171-8588 (Japan)
E-mail: hiroyuki.nakamura@gakushuin.ac.jp

[**] This work was partially supported by a Grant-in-Aid for Scientific Research on Innovative Areas "Chemical Biology of Natural Products" from The Ministry of Education, Culture, Sports, Science and Technology (Japan).

Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/anie.201303831.

We first examined TRTs suitable for the SET-based addition reaction using angiotensin II as the model peptide. Among the compounds examined, N′-acetyl-N,N-dimethyl-1,4-phenylenediamine (**1**; Table 1) was found to be the most suitable TRT for this reaction, and the monoadduct to angiotensin II was obtained in 50% yield in the presence of the $[Ru(bpy)_3]Cl_2$ complex in Tris buffer (10 mM, pH 4.2) and under irradiation with light (Table S1 in the Supporting Information). Then, we tested different buffers and additives for the addition reaction with TRT **1** under mild conditions (pH 6.0–7.4) to prevent proteins from denaturation (Table 1 and Table S2 in the Supporting Information). The addition reaction proceeded preferentially in 2-morpholinoethanesulfonic acid (10 mM, MES) buffer under irradiation with visible light for 15 min (Table 1, entry 1). It was reported that SET of the excited state $*Ru^{II}$ to Ru^{III} was accelerated in the presence of oxidants, such as ammonium persulfate (APS).[21] Therefore, we examined the effects of APS on the addition reaction of angiotensin II (100 μM) with **1** (500 μM). The reaction proceeded smoothly at pH 7.4 in the presence of APS (1 mM) and a catalytic amount of $[Ru(bpy)_3]Cl_2$ (10 μM) under irradiation with visible light for 5 min, giving the monoadduct and the bisadduct (9:1 ratio) in 70% yield (Table 1, entry 2). The use of 1 mM of $[Ru(bpy)_3]Cl_2$ accelerated the reaction rate and both mono- and bisadducts were obtained in 95% yield in a 3:2 ratio even under irradiation with visible light for 1 min (Table 1, entry 3). The reaction was abolished by addition of dithiothreitol (DTT), a radical scavenger (Table 1, entry 4). Both irradiation with visible light and the $[Ru(bpy)_3]Cl_2$ complex were essential for the addition reaction (Table 1, entries 5–7),[23] thus suggesting that the reactions proceeded through the photoinduced oxidative SET radical mechanism proposed in Scheme S1 in the Supporting Information. MS/MS analysis of the mono- and bisadducts of **1** to angiotensin II indicated that all modifications were implemented on tyrosine residues (Figures S1 and S2 in the Supporting Information). To understand the binding mode between **1** and tyrosine residues, the addition reaction of **1** with ethyl-N-acetyl-tyrosine amide, a model substrate, was investigated. Structural analysis revealed that a carbon–carbon bond was formed between the *ortho*-carbon atom of the phenolic oxygen of ethyl-N-acetyl-tyrosine amide and the *ortho*-carbon atom of the dimethylamino group of **1** (Figures S3 and S4 in the Supporting Information). We also examined the reactivity of **1** to the tryptophan residue by using the model peptide melittin. The observed oxidation of the tryptophan residue with oxygen proceeded prior to the addition with **1**.[24–26] These results suggest that this method can be used for the specific modification of tyrosine residues in the target protein.

Table 1: Optimization of the addition reaction of angiotensin II with N′-acetyl-N,N-dimethyl-1,4-phenylenediamine (**1**).[a]



Entry	$[Ru(bpy)_3]Cl_2$	Buffer pH	Irradiation time [min]	Additive	Yield [%] (mono-/bisadduct)
1	1 mM	6.0	15	–	55 (1:0)
2	10 μM	7.4	5	1 mM APS	70 (9:1)
3	1 mM	7.4	1	1 mM APS	95 (3:2)
4	1 mM	7.4	1	1 mM APS 10 mM DTT	0
5	1 mM	7.4	–[b]	1 mM APS	10 (1:0)
6	–	7.4	–[b]	1 mM APS	10 (1:0)
7	1 mM	7.4	–[b]	–	0

[a] Reaction conditions: angiotensin II (100 μM) and **1** (500 μM) in MES (10 mM) buffer. All reactions were quenched with DTT (10 mM) and analyzed by using MALDI-TOF MS. Each reaction was repeated several times and the average ratios of the mono- and bisadducts are indicated. [b] Incubated for 5 min without irradiation.

With the suitable conditions established, we applied this tyrosine-residue-specific addition reaction to the modification of a purified protein. We designed and synthesized fluorescent TRT **2** (Scheme S3 in the Supporting Information). Bovine serum albumin (BSA), the model protein, was treated with **2** under several conditions (Figure 2). Whereas BSA was not modified in the presence of $[Ru(bpy)_3]Cl_2$ without **2** or APS (lanes 1 and 2), successful modification was observed in the presence of $[Ru(bpy)_3]Cl_2$ (10 μM), **2** (500 μM), and APS (1 mM) under irradiation with visible light for 5 min (lane 3). A slight modification of BSA with **2** was observed without irradiation and $[Ru(bpy)_3]Cl_2$ (lanes 4 and 5), thereby revealing that APS caused a photocatalyst-independent modification reaction, which was considered to be one of the undesired background reactions. This modification proceeded without APS, although an excess amount of [Ru-

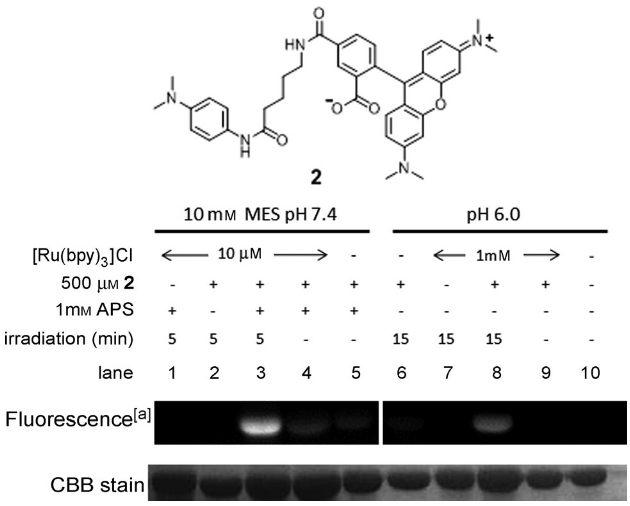


Figure 2. Modification of BSA with fluorescent TRT **2** under various conditions. Fluorescence images and coomassie brilliant blue (CBB)-stained images of SDS-PAGE gels, with the conditions for each lane given above. [a] The exposure time for fluorescence detection in lanes 6–10 was longer (3.0 s) than that in lanes 1–5 (0.3 s; also see Figure S7 in the Supporting Information).

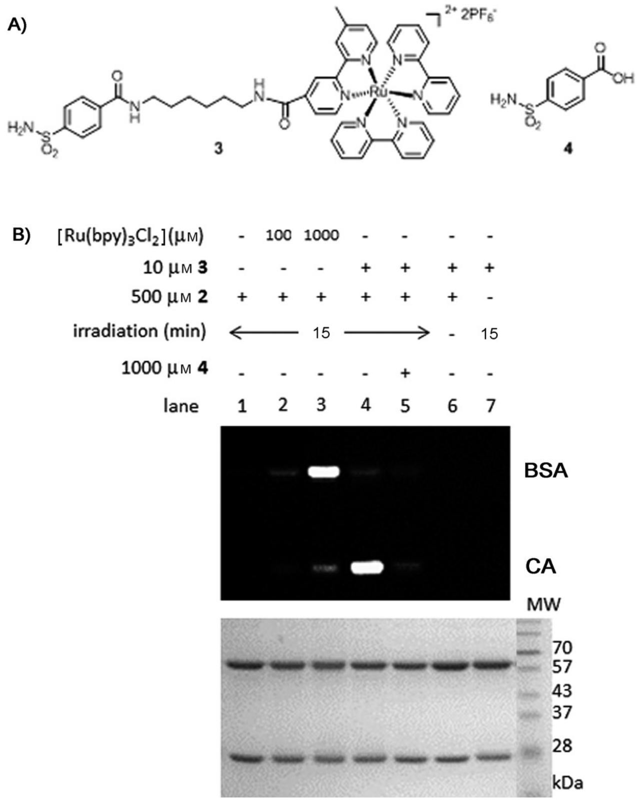


Figure 3. Selective modification of carbonic anhydrase (CA) promoted by the CA-ligand-conjugated ruthenium complex **3** as LSC in a mixture of BSA and bovine CA. A) Structures of **3** and CA-binding competitor **4**. B) Protein modification reactions were performed in a mixture of BSA (5 μM) and CA (5 μM) in MES buffer (10 mM; pH 6.0) without APS. Fluorescence images of modified proteins (upper) and whole proteins with CBB stain (lower) are shown.

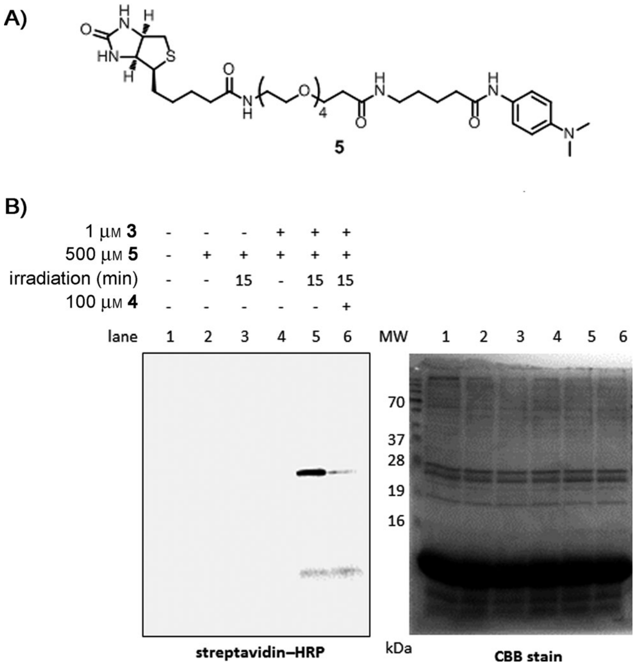


Figure 4. Selective modification of CA in mouse erythrocyte lysates. A) Structure of biotin-conjugated TRT **5**. B) The reaction for the selective modification of CA was performed in MES (10 mM, pH 6.0) using erythrocyte lysates from 6-week-old female mice. The left image shows visualization of biotinylated proteins with a streptavidin–HRP conjugate. The right image shows CBB staining of proteins in mouse erythrocyte lysates.

(bpy)$_3$]Cl$_2$ (1 mM) was needed. [Ru(bpy)$_3$]Cl$_2$ and irradiation with light were indispensable in this case (lanes 6–10). To evaluate the biocompatibility of this reaction, double modification experiments of BSA and streptavidin were carried out using PEG-conjugated TRT and tetramethylrhodamine-conjugated maleimide or *N*-hydroxysuccinimide (NHS). The electrophilic modifications proceeded even after the PEG modification, thus suggesting that this SET-based tyrosine-targeting method is compatible with the electrophilic methods targeting cysteine and lysine residues (see Figure S8 in the Supporting Information).

To clarify whether this addition reaction based on local SET is applicable to selective protein modification, we designed and synthesized a ligand-conjugated ruthenium complex as an LSC. Carbonic anhydrase (CA) and benzenesulfonamide were chosen as the model of the target protein and its ligand, respectively.[27] When the SET-based radical modification reaction was carried out in the presence of [Ru(bpy)$_3$]Cl$_2$ using a mixture of BSA and CA, the extent of modification of BSA was higher than of CA, because it had a larger number of accessible tyrosine residues (Figure 3, lanes 2 and 3). Meanwhile, benzenesulfonamide-conjugated ruthenium complex **3**, which was designed as a CA-targeting LSC, promoted the selective modification of CA (lane 4) with more than 100 times higher efficiency than BSA modification (lane 3; 1 mM [Ru(bpy)$_3$]Cl$_2$ versus lane 4; 10 μM **3**).[28] The selective modification of CA was inhibited by the CA-binding competitor **4**, which was present in an amount that was 100 times that of LSC **3** (lane 5), thereby revealing that this reaction was accelerated by the target protein–ligand interaction between CA and **3**.[29] No protein modification of both BSA and CA was observed in the absence of irradiation with visible light or **2** (lanes 6 and 7).

Finally, we applied this target-selective protein modification to native CA proteins in cell lysate using **3**. CA modification was performed in mouse erythrocytes that originally expressed CAs. However, the lysates of erythrocytes contained a large amount of hemoglobin (14 kDa) and the fluorescence of hemoglobin interfered with the fluorescence detection of CA modified with **2**. Therefore, we developed biotin-conjugated *N*,*N*-dimethyl-1,4-phenylenediamine **5** as a TRT (Figure 4 A) to detect the target protein by using a streptavidin–horseradish peroxidase(HRP) conjugate. No biotinylated proteins were detected with streptavidin–HRP in the absence of **3**, biotin-conjugated TRT **5**, or irradiation (Figure 4B, lanes 1–4). Those proteins, however, were observed at 29 kDa, which corresponded to CA from the cell lysate, in the presence of **3** and **5** under irradiation with light for 15 min (lane 5), and this modification was inhibited by the addition of **4** (lane 6).[30] These results clearly indicated that SET-based protein modification using **3** and **5** specifically occurred on the target protein CA despite the coexistence of various kinds of proteins in the cell lysate. We also performed this modification reaction in intact erythrocytes in MES-buffered saline (10 mM MES (pH 6.0), 150 mM NaCl) and again obtained similar results to those from the cell lysate (Figure S11 in the Supporting Information), thus suggesting that **5** and **3** could permeate the erythrocyte cell membrane.[31] Therefore, this target-selective protein modification based on local SET catalysis is applicable to the modification of native proteins in an intracellular environment.

In conclusion, we have developed a target-selective protein modification strategy based on local SET catalysis. In cell lysates, tyrosyl radicals were selectively generated on the target protein by SET from the photocatalyst in the local environment of the ligand, and the radicals were trapped by TRTs through the specific radical addition to tyrosine residues. Although target-selective protein modifications in cell lysates were achieved before with electrophilic reagents using ligand-tethered DMAP catalysts, those methods were limited to modifying the nucleophilic residues of the targeted proteins, such as lysine and cysteine. The current method is not only an alternative to the strategy using electrophilic agents but can also be utilized for the identification of proteins interacting with the target protein through a tyrosine–tyrosine SET addition reaction between the target protein and its interaction partners induced by the LSC-based generation of tyrosyl radicals. Studies with respect to further applications are in progress.

Received: May 4, 2013

Keywords: photocatalysts · protein modifications · radical reactions · single-electron transfer · tyrosine

---

[1] T. Hayashi, I. Hamachi, *Acc. Chem. Res.* **2012**, *45*, 1460 – 1469.

[2] A. M. Wu, P. D. Senter, *Nat. Biotechnol.* **2005**, *23*, 1137 – 1146.

[3] J. M. McFarland, M. B. Francis, *J. Am. Chem. Soc.* **2005**, *127*, 13490 – 13491.

[4] G. J. L. Bernardes, J. M. Chalker, J. C. Errey, B. G. Davis, *J. Am. Chem. Soc.* **2008**, *130*, 5052 – 5053.

[5] A. Dondoni, *Angew. Chem.* **2008**, *120*, 9133 – 9135; *Angew. Chem. Int. Ed.* **2008**, *47*, 8995 – 8997.

[6] J. M. Antos, M. B. Francis, *J. Am. Chem. Soc.* **2004**, *126*, 10256 – 10257.

[7] S. D. Tilley, M. B. Francis, *J. Am. Chem. Soc.* **2006**, *128*, 1080 – 1081.

[8] J. M. Antos, J. M. McFarland, A. T. Iavarone, M. B. Francis, *J. Am. Chem. Soc.* **2009**, *131*, 6301 – 6308.

[9] N. S. Joshi, L. R. Whitaker, M. B. Francis, *J. Am. Chem. Soc.* **2004**, *126*, 15942 – 15943.

[10] H. Ban, J. Gavrilyuk, C. F. Barbas, *J. Am. Chem. Soc.* **2010**, *132*, 1523 – 1525.

[11] K. L. Seim, A. C. Obermeyer, M. B. Francis, *J. Am. Chem. Soc.* **2011**, *133*, 16970 – 16976.

[12] J. Gavrilyuk, H. Ban, M. Nagano, W. Hakamata, C. F. Barbas, *Bioconjugate Chem.* **2012**, *23*, 2321 – 2328.

[13] B. V. Popp, Z. T. Ball, *J. Am. Chem. Soc.* **2010**, *132*, 6660 – 6662.

[14] Z. Chen, B. V. Popp, C. L. Bovet, Z. T. Ball, *ACS Chem. Biol.* **2011**, *6*, 920 – 925.

[15] S. Tsukiji, M. Miyagawa, Y. Takaoka, T. Tamura, I. Hamachi, *Nat. Chem. Biol.* **2009**, *5*, 341 – 343.

[16] S.-h. Fujishima, R. Yasui, T. Miki, A. Ojida, I. Hamachi, *J. Am. Chem. Soc.* **2012**, *134*, 3961 – 3964.

[17] Y. Koshi, E. Nakata, M. Miyagawa, S. Tsukiji, T. Ogawa, I. Hamachi, *J. Am. Chem. Soc.* **2008**, *130*, 245 – 251.

[18] H. Wang, Y. Koshi, D. Minato, H. Nonaka, S. Kiyonaka, Y. Mori, S. Tsukiji, I. Hamachi, *J. Am. Chem. Soc.* **2011**, *133*, 12220 – 12228.

[19] N. Hoffmann, *Chem. Rev.* **2008**, *108*, 1052 – 1103.

[20] K. Zeitler, *Angew. Chem.* **2009**, *121*, 9969 – 9974; *Angew. Chem. Int. Ed.* **2009**, *48*, 9785 – 9789.

[21] D. A. Fancy, T. Kodadek, *Proc. Natl. Acad. Sci. USA* **1999**, *96*, 6020 – 6024.

[22] K. Kim, D. A. Fancy, D. Carney, T. Kodadek, *J. Am. Chem. Soc.* **1999**, *121*, 11896 – 11897.

[23] The modifications induced by APS were observed in 10% conversion yield without irradiation with light and/or [Ru(bpy)$_3$]Cl$_2$ in entries 5 and 6, respectively.

[24] R. T. Dean, S. Fu, R. Stocker, M. J. Davies, *Biochem. J.* **1997**, *324 (Pt 1)*, 1 – 18.

[25] M. J. Davies, *Biochim. Biophys. Acta Proteins Proteomics* **2005**, *1703*, 93 – 109.

[26] M. J. Davies, *Biochem. Biophys. Res. Commun.* **2003**, *305*, 761 – 770.

[27] V. M. Krishnamurthy, G. K. Kaufman, A. R. Urbach, I. Gitlin, K. L. Gudiksen, D. B. Weibel, G. M. Whitesides, *Chem. Rev.* **2008**, *108*, 946 – 1051.

[28] The use of lower amounts of compound **3** resulted in lower labeling efficiency owing to the tight binding of compound **3** to CA.

[29] We examined the CA inhibitory activity of compounds **3**, **4** and [Ru(bpy)$_3$]Cl$_2$ according to the literature protocol reported by J. A. Verpoorte, S. Mehta, J. T. Edsall, *J. Biol. Chem.* **1967**, *242*, 4221 – 4229. Compound **3** showed higher CA inhibitory activity (IC$_{50}$: 0.50 μM) than compound **4** (IC$_{50}$: 1.6 μM). [Ru(bpy)$_3$]Cl$_2$ did not show a significant inhibition at 10 μM. See Figure S9 in the Supporting Information.

[30] We examined the selective modification of CA in mouse erythrocyte lysates at various concentrations of LSC **3** and found that the selectivity of the modification at 1 μM was higher than that at 10 μM, although the modification efficiency dropped. Therefore, we carried out the further CA-selective modification experiments in mouse erythrocyte lysates at 1 μM of **3**.

[31] Q. Shao, B. Xing, *Chem. Commun.* **2012**, *48*, 1739 – 1741.