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Bioconjugation with Thiols by Benzylic Substitution

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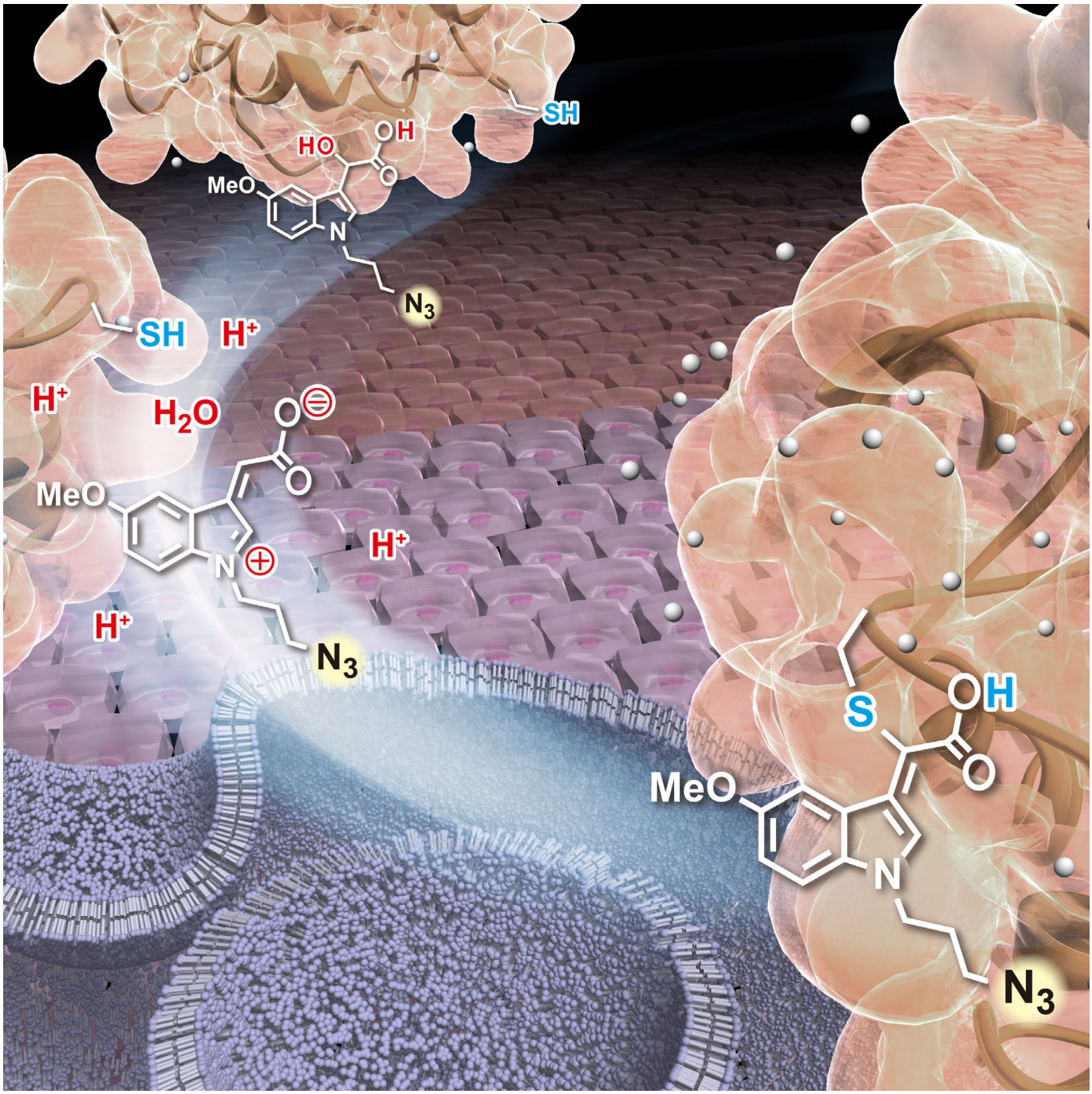

Abstract: A benzylic substitution of 3-indolyl(hydroxyl)-acetate derivatives with thiols proceeded specifically in the presence of amino, carboxy, and phosphate groups in weakly acidic aqueous solutions under nearly physiological condition, while no reaction occurred at pH over 7. Kinetic studies revealed that the reaction followed second-order kinetics (first-order in the reactant and first-order in thiol) in contrast with the S_N1 mechanism of common benzylic substitution of alcohols. The utility of the present method for functionalization of biomacromolecules was demonstrated using several model proteins, such as lysozyme, insulin, trypsin, and serum albumin. The catalytic bioactivity of lysozyme in lysis of Micrococcus lysodeikticus cells was completely retained after the modification.

Conjugation between natural functional groups in biomolecules and synthetic compounds has various applications, such as the synthesis of biologic tools for elucidating biofunctions of living organisms in situ,[1] preparing antibody-drug conjugates,[2] and constructing tailor-made libraries of bioactive molecules.[3] Therefore, many scientists are interested in these reactions, but the development of suitable reactions for bioconjunction remains difficult. Criteria for bioconjugation are (i) sufficient reactivity in aqueous medium at mild temperature, (ii) functional group selectivity/tolerance, (iii) high regioselectivity, and (iv) safety of reagents and co-products formed during the reaction.[4,5] These requirements limit the reaction types applicable for bioconjugation.

Cysteine is often used as a target for selective bioconjugation because it has a reactive thiol group, except when forming a disulfide bond.[5] Michael addition of thiol to α,β-unsaturated carbonyl[6]/sulfone,[7] substitution of thiol with haloalkane[8]/haloarene[9]/organopalladium[10] compounds, thiol-ene,[11] thiol-yne[12] and disulfide exchange[13] are representative reaction types used in this field. The Michael addition and substitution reactions are often carried out at pH 6.5–7.5[14] and pH 7.2–9.0,[4] respectively (Figure 1 a). Although, the reactions are well established and have already been applied to the functionalization of bioactive peptides and protein conjugates,[5,15] concerns regarding the exchange of thiol in the maleimide adduct, oxidation of thiol to disulfide, and competition with amine nucleophile at high pH still remain.

Our research group has been studying the direct catalytic activation of allylic alcohol through π-allyl metal formation.[16] During the course of our studies,[17] we realized that hydroxyl

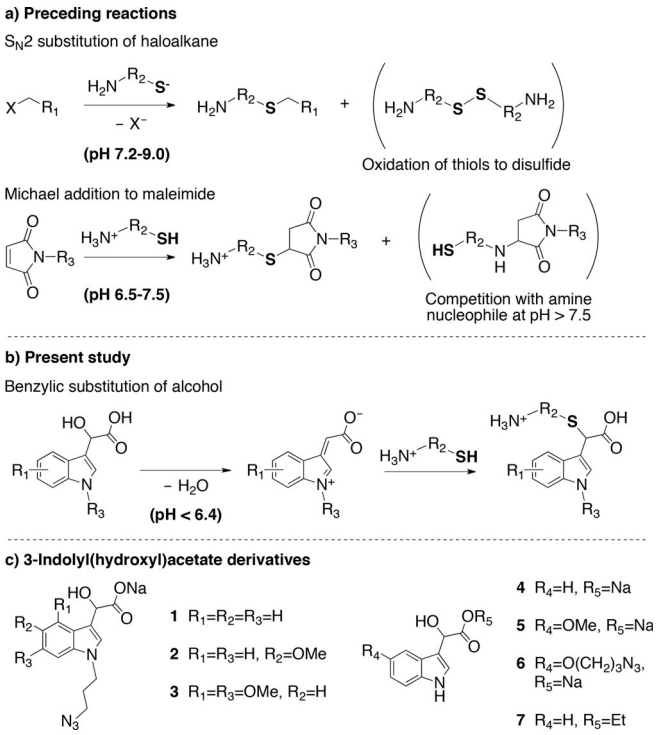

Figure 1. Preceding reactions used in bioconjugation (a), present study (b) and 3-indolyl(hydroxyl)acetates having reactive benzylic C–O bonds (c).

groups at the benzylic position can be activated by an action of Lewis and Brønsted acids,[18] and substitution reactions by nucleophiles proceeded via carbocation intermediates[19] Thus, we hypothesized that benzylic substitutions of appropriately designed reactants could be used for a selective bioconjugation with thiol at a weakly acidic pH under nearly physiological conditions (Figure 1 b). Since this reaction, involving the activation of benzylic hydroxyl group, proceeds under weakly acidic conditions, the applicable pH range differs from those of the preceding reactions (Figure 1 a). Moreover, since this reaction gives water as the only co-product, and does not use heavy metal, light irradiation, or radical initiator, it is highly biocompatible.

Screening of several substrates with a reactive C–O bond at the benzylic position (see Table S1 in the Supporting Information) revealed that the class of water-soluble indole derivatives shown in Figure 1 c was the most reactive against thiol nucleophiles. These indole derivatives were synthesized from commercially available starting materials in 1–7 steps (see the Supporting Information).

Figure 2 shows the ^{1}H NMR spectra of the reaction mixtures of 3-indolyl(hydroxyl)acetate **2** (0.010 M) and glutathione in sodium phosphate (0.20 M) D_2O solutions at the indicated pD for 37 °C (after 4 h). At pD 7.4 with 1.2 equivalents of glutathione, almost no changes in the aromatic and benzylic signals of **2** were observed (Figure 2 a) compared with the signals of **2** in D_2O without glutathione (Figure 2 d), suggesting that **2** did not react with glutathione at pD 7.4. Full conversion of **2** and new signals attributed to the thiol-adduct **8** were observed in the pD 5.4 solution (Figure 2 b; see the Supporting Information

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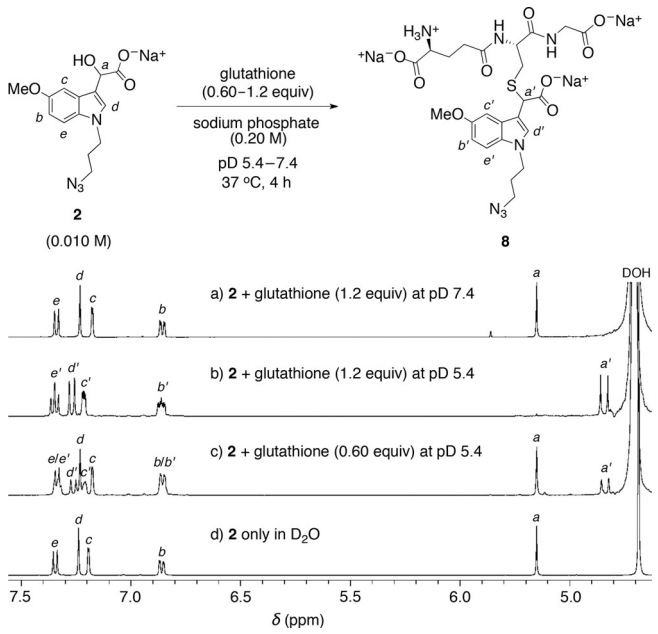

Figure 2. NMR spectra of reaction mixtures of **2** (0.010 M) and glutathione in sodium phosphate (0.20 M) D_2O solutions at 37 °C for 4 h. a) **2** + glutathione (1.2 equiv) at pD 7.4. b) **2** + glutathione (1.2 equiv) at pD 5.4. c) **2** + glutathione (0.6 equiv) at pD 5.4. d) **2** only in D_2O. The aromatic and benzylic peaks were shown for clarity. The reaction mixtures were neutralized with $NaDCO_3$ before the NMR measurements.

for characterization of **8**). When **2** was reacted with 0.6 equivalents of glutathione, a mixture of **8** and unreacted **2** was observed (Figure 2c), suggesting that **2** selectively reacted with thiol and was intact against the amino and carboxylate nucleophiles of the peptide. The reaction at pD 4.0 proceeded smoothly, similar to that at pD 5.4 (see Figure S1 in the Supporting Information). 3-indolyl(hydroxyl)acetate **2** remained intact against both amine and thiol nucleophiles even at pD 9.5 (Figure S1), suggesting that the activation of benzylic hydroxyl group was essential in the reaction. Exchange of thiol was not observed by the treatment of **8** with 10 equivalents of 2-mercaptoethanol at pD 5.4 and 37 °C, indicting a high stability of the thioether linkage under physiological conditions (see FigureS2 in the Supporting Information). In addition, **2** itself was stable at pD 5.4 (no decomposition).

Next, the kinetics of the reaction between **2** (0.00010 M) and glutathione (0.0050 M) were studied in a UV/Vis experiment (Figure 3a). Systematic spectral changes with the existence of isosbestic points indicated clean consumption of **2**, even in diluted conditions. When excess amounts of glutathione were present (50 equiv), the concentration of glutathione during the reaction can be considered as steady-state, and the reaction was assumed to be a pseudo first-order reaction [Eqs. (S1), (S2) in the Supporting Information].

Non-linear fitting using Eq. (S2) to the absorbance changes showed excellent agreement ($R^2 = 0.9998$) and supported a second-order reaction (Figure 3b). The reaction orders with re-

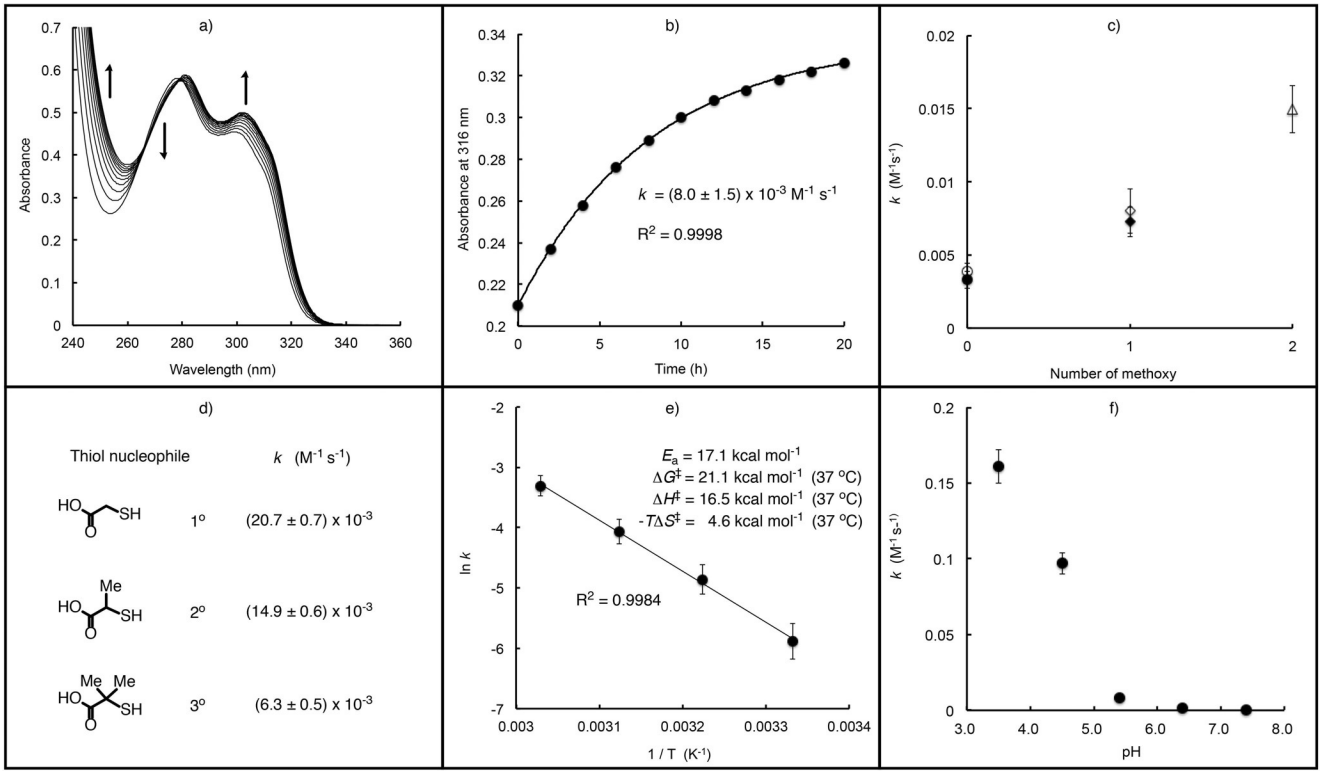

Figure 3. a) UV/Vis spectral changes of **2** (0.00010 M) upon reaction with glutathione (0.0050 M) in a pH 5.4 sodium phosphate (0.20 M) aqueous solution at 37 °C. The spectra were recorded at 2 h interval. b) Non-linear fitting of absorbance changes of **2** at 316 nm to Eq. (2). c) Correlation between *k* values and number of methoxy on indole: ○ = **1**, ◇ = **2**, △ = **3**, ● = **4**, ◆ = **5**. d) Steric effect of thiol nucleophiles. e) Eyling plot of the reaction between **2** and glutathione at varied temperatures (27, 37, 47 and 57 °C). f) Plots of *k* values at varied pH (pH 3.5, 4.5, 5.4, 6.4 and 7.4).

spect to **2** and glutathione were 1.09 and 0.93 (sum = 2) as determined from the relationship between the initial rate and the substrate concentrations (see Figure S3 and S4 in the Supporting Information). Interestingly, although benzylic substitution of alcohols generally proceeded through an S_N1 mechanism, where formation of the carbocation intermediates is the rate-determining step,[19e] the present benzylic substitution reaction had a second-order dependency on the concentration of both substrates, indicating that the reaction between the iminium cations and thiols are the rate-determining step (Figure 1 b). To the best of our knowledge, this is the first example of a second-order reaction of benzylic substitution of alcohols in homogeneous aqueous media.

Similar experiments were performed with various 3-indolyl(hydroxyl)acetates **1**–**7**, and the rate constants (k) of those substrates are summarized in Table 1. The k values increased depending on the number of methoxy groups on the indole (Figure 3 c), indicating that the electron density of the indole aromatic systems influenced the reactivity (Table 1, entries 1–5). When the number of methoxy groups was the same, N-alkylated and NH-free indole-substrates had similar k values (entry 1 vs. 4, entry 2 vs. 5), indicating that the N-alkylation of indole had a minor influence on the reaction rate. When 1°, 2° and 3° thiols were used as the nucleophiles, the k values decreased with increasing steric congestion, but very importantly, even 3° thiols had high reactivity (Figure 3 d).

Table 1. Rate constants (k) of substrates **1**–**7** and calculated electron density of the aromatic systems.

Entry	Substrate	k [$M^{-1}s^{-1}$][a]	Electron density
1	**1**	$3.9\pm0.5\times10^{-3}$	−1.507
2	**2**	$8.0\pm1.5\times10^{-3}$	−0.976
3	**3**	$15.0\pm1.6\times10^{-3}$	−0.437
4	**4**	$3.3\pm0.6\times10^{-3}$	−1.537
5	**5**	$7.3\pm1.0\times10^{-3}$	−1.006
6	**6**	$8.8\pm0.9\times10^{-3}$	−1.006
7	**7**	no reaction	−1.588
8[b]	**2**	$7.4\pm1.3\times10^{-3}$	−0.976
9[c]	**2**	$1.5\pm0.1\times10^{-3}$	−0.976
10[d]	**2**	no reaction	−0.976

[a] Average of three experiments (± standard error). [b] pH 5.4 sodium acetate. [c] pH 6.4 sodium phosphate. [d] pH 7.4 sodium phosphate.

Almost the same k values were obtained when the reaction was performed in sodium acetate solution instead of a sodium phosphate solution at pH 5.4 (Table 1, entry 2 vs. 8). Although large excess amounts of phosphate or acetate anion existed against **2** (2000 equiv), counteranions in the buffer solution seemed to have minor effects on the reaction. Substrate **2** remained reactive at pH 6.4 (entry 9; 4.1-fold reduced rate), while the reactivity completely diminished at pH 7.4 (entry 10), consistent with the results of NMR studies.

Figure 3 e shows the Eyring plot obtained from the k values of **2** at various temperatures (27 to 57 °C). The activation parameters E_a, $\Delta G^{\ddagger}$, $\Delta H^{\ddagger}$, and $-T\Delta S^{\ddagger}$ at 37 °C were determined to be 17.1, 21.1, 16.5, and 4.6 kcal mol^{-1}, respectively, and the $\Delta H^{\ddagger}$ term was the predominant requirement for the activation. Interestingly, the steep increase in the k values was observed at the pH below 4.5 (Figure 3 f). In addition, ethyl 2-hydroxy-2-(1*H*-indol-3-yl)acetate (**7**), which has an ester instead of a carboxylate, exhibited no reactivity at pH 5.4 (Table 1, entry 7); the carboxy moiety was essential for the reaction.[18c]

The utility of the present reaction for site-selective bioconjugation was studied using lysozyme (chicken egg-white) as a model protein. Lysozyme is a 14.3 kDa enzyme that catalyzes the hydrolysis of peptidoglycans on bacterial cell walls. The enzyme contains four disulfide bridges, and the C6–C127 bridge is exposed on the protein surface, while the other three bonds are buried inside.[20] Although cysteine can be mutagenetically introduced to proteins,[21] thiols derived from reduction of native disulfide are also often use for bioconjugation.[22] Reduction of native enzymes with 1.0 equivalent of tris(2-carboxyethyl)phosphine hydrochloride (TCEP)[22] at pH 4.5, and the following one-pot addition of **2** afforded bioconjunction compound **9** (Figure 4). ESI-TOF MS of **9** showed m/z = 1461.2,

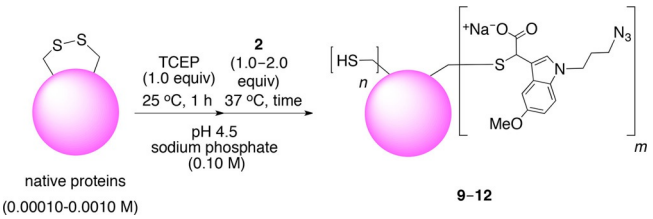

Figure 4. Preparation of protein conjugates **9**–**12**.

which corresponds to $[M+10H]^{10+}$ of **9**, in which one unit of **2** was introduced to the enzyme (Table 2, entry 1). The amount of **2** introduced to the protein was determined to be m = 1.03 based on the absorbance of 5-methoxyindole at 316 nm (ε = 3180 M^{-1}cm^{-1}; see the Supporting Information). The amount of remaining thiol in the product was quantified by Ellman's

Table 2. ESI-TOF MS of native and modified proteins (**9**–**12**). Reaction time and amounts of **2** (m) and thiol (n) in the products.

Entry	Protein	Calculated m/z	Found m/z	Time [h]	Product	Calculated m/z	Found m/z	m	n
1[a]	lysozyme	$[M+10H]^{10+}$ = 1432.4	1432.4	15	**9**	$[M+10H]^{10+}$ = 1461.2	1461.2	1.03	0.94
2[b]	insulin	$[M+5H]^{5+}$ = 1160.1	1160.1	3	**10**	$[M+5H]^{5+}$ = 1217.8	1217.8	0.95	0.85
3[b]	trypsin	$[M+17H]^{18+}$ = 1295.7	1295.7	15	**11**	$[M+17H]^{18+}$ = 1311.7	1311.7	0.89	0.93
4[c]	serum albumin	$[M+47H]^{47+}$ = 1414.4	1414.4	15	**12**	$[M+47H]^{47+}$ = 1420.5	1420.5	0.65	0.40

[a] [protein] = 0.0001 M, [**2**] = 0.0002 M (2 equiv). [b] [protein] = 0.0001 M, [**2**] = 0.0001 M (1 equiv). [c] [protein] = 0.001 M, [**2**] = 0.002 M (2 equiv).

test (Table 1, $n = 0.94$).[13c] Oxidation of thiol to disulfide was not observed under the weakly acidic pH. The second-order rate constant of the reaction between **2** and reduced lysozyme was determined to be $0.13 \pm 0.01\ M^{-1} s^{-1}$ (see Figure S5 in the Supporting Information), which was moderate compared to that of maleimide ($730\ M^{-1} s^{-1}$). The peptides containing the C6 and C127 residues functionalized with **2** were found from the tryptic digestion of **9** (see the Supporting Information).

The generality of the present reaction for functionalization of biomacromolecules was studied with several proteins having various molecular weights: human insulin (5.8 kDa),[23] bovine trypsin (23.3 kDa),[24] and bovine serum albumin (66.4 kDa).[25] The results are summarized in Table 2. Insulin has low solubility at neutral pH, but it is soluble in acidic aqueous solutions. The disulfide bridge between C7 (A chain) and C7 (B chain) is known to be the most exposed to solvent.[23b] Reaction of reduced insulin with **2** at pH 4.5 gave product **10** (Figure 4), as confirmed by ESI-TOF MS (Figure 4; Table 1, entry 2).

Trypsin is a serine protease that shows catalytic activity at basic pH conditions (pH 8–9). Functionalization of trypsin at this pH range is problematic because of autodigestion of the enzyme. Successful protein modification at pH 4.5 was confirmed (entry 3). Bovine serum albumin (BSA) has a reactive thiol at Cys34,[25b] which is often used as a target for surface modification of the protein. BSA treated with TCEP (1 equiv) reacted moderately with **2** ($m = 0.65$, entry 4). The modification at Cys34 was confirmed by tryptic digestion of **12** (see the Supporting Information).

The catalytic bioactivity of modified lysozyme (**9**) in lysis of Micrococcus lysodeikticus cells was studied (see Figure S6 in the Supporting Information). The modified lysozyme completely retained the bioactivity compared to the native enzymes. Circular dichroic (CD) spectra of the modified and native enzymes showed no significant changes (see Figure S7 in the Supporting Information).

In conclusion, we developed an environmentally responsive/friendly bioconjugation reaction that did not require additional hazardous reagents, oxidants or heavy metals. The bioconjunction compounds with **2** can be used as a platform for further functionalization via an alkyne–azide cycloaddition reaction (see Figures S8–S9 in the Supporting Information).[26] Chemical systems that are responsive at specific pH environments have various applications such as drug delivery,[27] protein engineering,[28] cell engineering,[29] cell imaging,[30] and elucidation of biofunctions of thiol-containing bioactive molecules.[31] Human bodies are maintained at neutral conditions, but specific organs and regions, such as lysosomes (pH 4.5–5.0), endosomes (pH 5.5–6.0), digestive organs (pH 1.5–6.8), and exterior surfaces of cancerous cells, are in an acidic state.[31] Further applications of the present methodology are ongoing in our laboratory.

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Conflict of interest

The authors declare no conflict of interest.

Keywords: bioconjugation · nucleophilic substitution · pH-responsive · protein modifications · sulfur

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