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Novel Probes Showing Specific Fluorescence Enhancement on Binding to a Hexahistidine Tag

Mie Kamoto, Naoki Umezawa,* Nobuki Kato, and Tsunehiko Higuchi*[a]

Abstract: The introduction of hexahistidine (His tag) is widely used as a tool for affinity purification of recombinant proteins, since the His tag binds selectively to nickel–nitrilotriacetic acid (Ni^{2+}–NTA) complex. To develop efficient "turn-on" fluorescent probes for His-tagged proteins, we adopted a fluorophore displacement strategy, that is, we designed probes in which a hydroxycoumarin fluorophore is joined via a linker to a metal–NTA moiety, with which it forms a weak intramolecular complex, thereby quenching the fluorescence. In the presence of a His tag, with which the metal–NTA moiety binds strongly, the fluorophore is displaced, which results in a dramatic enhancement of fluorescence. We synthesized a series of hydroxycoumarins that were modified by various linkers with NTA (NTAC ligands), and investigated the chemical and photophysical properties of the free ligands and their metal complexes. From the viewpoint of fluorescence quenching, Ni^{2+} and Co^{2+} were the best metals. Fluorescence spectroscopy revealed a 1:1 binding stoichiometry for the Ni^{2+} and Co^{2+} complexes of NTACs in pH 7.4 aqueous buffer. As anticipated, these complexes showed weak intrinsic fluorescence, but addition of a His-tagged peptide (H-$(His)_6$-Tyr-NH_2; Tyr was included to allow convenient concentration measurement) in pH 7.4 aqueous buffer resulted in up to a 22-fold increase in the fluorescence quantum yield. We found that the Co^{2+} complexes showed superior properties. No fluorescence enhancement was seen in the presence of angiotensin I, which contains two nonadjacent histidine residues; this suggests that the probes are selective for the polyhistidine peptide.

Keywords: cobalt · fluorescent probes · molecular recognition · peptides · sensors

Introduction

For proteomics research, huge numbers of proteins must be characterized. The fluorescent labeling of proteins has proven to be a powerful approach for following the dynamic processes of protein synthesis and degradation, identifying localization, and studying protein–protein interactions, both in vitro and in vivo.[1] Many labeling techniques have been developed that are based on the reaction of fluorescent dyes bearing functional groups, such as succinimidyl ester or maleimide, that react with primary amines or thiols exposed on the protein surface. However, these techniques are typically nonspecific. The most commonly applied method for labeling a target protein selectively is by expressing the protein as a fusion with a fluorescent protein, such as green fluorescent protein (GFP). This approach has been used to study the dynamics of individual proteins in living cells.[2] Although this technique offers absolute specificity, there remain some limitations. The fluorescent protein requires a period of up to several hours to mature into a soluble, fluorescent protein, and this is too slow for some applications. The folding of fluorescent proteins is known to be problematic, especially at or above room temperature.[2] Also, the fluorescence of GFP is sensitive to environmental factors, such as pH, and can be difficult to distinguish from cellular autofluorescence.[2] GFP variants have been produced through protein engineering to overcome these limitations, but the steric bulk of this protein (at least 220 aa) still has the potential for significant perturbation of folding, trafficking, and function of proteins to which it is fused.[3]

Alternative protein-labeling strategies have emerged that address the steric bulk and stability problems, as well as the limited range of fluorescence characteristics of GFP and its variants. Representative techniques involve the introduction

[a] M. Kamoto, Dr. N. Umezawa, Dr. N. Kato, Prof. Dr. T. Higuchi
Graduate School of Pharmaceutical Sciences
Nagoya City University
3-1, Tanabe-dori, Mizuho-ku
Nagoya, 467-8603 (Japan)
Fax: (+81) 52-836-3435
E-mail: umezawa@phar.nagoya-cu.ac.jp
higuchi@phar.nagoya-cu.ac.jp

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of a short peptide that is able to react/interact specifically with a designed fluorescent molecule.[4,5] The combination of a short peptide tag (a specific target for the fluorescent probe) and a functional fluorescent molecule (a fluorescent probe that recognizes the tag peptide) has great potential, because technology is available for tagging proteins in a selective, site-specific, and reversible manner.[6,7] This approach has several advantages, for example, it reduces perturbation that is caused by attachment of the fluorophore and offers the possibility of using a wide variety of functional molecules, besides fluorophores, as labels.

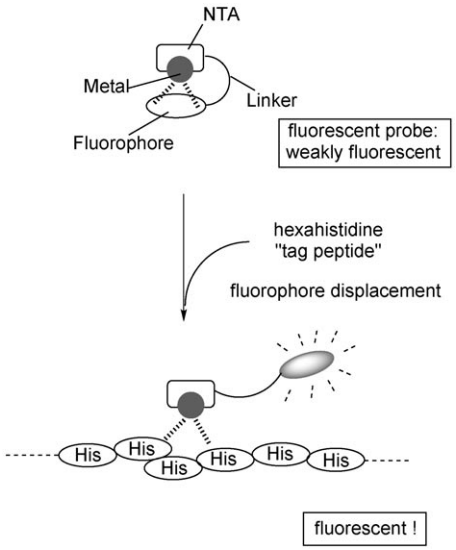
Tsien and co-workers have developed a technique that utilizes a tag peptide sequence containing four Cys residues positioned to allow reaction with a biarsenical fluorogenic compound.[6] The resulting biarsenical peptide adduct is more fluorescent than the initial fluorogenic compound. The tag peptide sequence necessary for reaction with the biarsenical molecule is small (16 amino acid residues in a typical application); this implies minimal perturbation of the target protein, and presents a minimal 4-Cys motif (CCPGCC) that confers adequate specificity. The main disadvantage is the toxicity of the arsenic derivatives used. Although Tsien has developed a protocol for the administration of antidotes that render the method broadly applicable, alternative methods that do not rely on such toxic metals would offer a distinct advantage.

Recently, several fluorescent derivatives of Ni^{2+}–nitrilotriacetic acid complex (Ni^{2+}–NTA), which targets the hexahistidine tag ($(His)_6$), have been reported.[8] The selective interaction between the His tag and Ni^{2+}–NTA has been widely used for the affinity purification of recombinant proteins, and the His tag itself is thought to have little influence on protein function.[9] However, most of the probes reported so far show no significant change in fluorescence intensity when the probe binds to the His tag, so detection requires fluorescence anisotropy experiments,[8d,e] or the use of the fluorescence resonance energy transfer technique with another fluorophore, such as GFP.[8a] More accurate and simpler monitoring of proteins would be possible if the probe showed fluorescence enhancement upon binding to the target protein. As far as we know, the only example of such a probe so far is dansyl–NTA–Ni^{2+} complex,[10] which uses the interaction between the field-sensitive dansyl group as a fluorophore and an additional hydrophobic tritryptophan motif coupled with the hexahistidine tag. This method is elegantly designed, but the position of the hexahistidine tag is limited to hydrophilic regions of the target protein and the environment of the labeled protein affects the fluorescence.

In this paper, we describe the design and synthesis of a series of His-tag-targeting fluorescent probes based on the hydroxycoumarin fluorophore. The design employs a fluorophore displacement strategy, that is, displacement of the fluorophore from a weak intramolecular complex with the His-tag-targeting moiety in the presence of His tag results in a large fluorescence enhancement. The tag sequence can be incorporated at any position of the target protein. Here, we describe the synthesis, chemical properties, and photophysical properties of a series of hydroxycoumarins modified by various linkers with NTA. We confirmed that the Co^{2+} complexes of these probes showed substantial fluorescence enhancement upon binding to hexahistidine, as expected.

Results and Discussion

Strategy for protein labeling with fluorescence enhancement: As a specific interaction between tag peptide and fluorescent probe, we focused on the interaction of a hexahistidine sequence with a metal complex. It is well-known that the Ni^{2+}–NTA or Co^{2+}–NTA complex interacts selectively with the hexahistidine sequence in aqueous media, by means of coordination of the imidazoles of the histidine side chains to the metal ion. Our strategy is summarized in Scheme 1. To control the fluorogenic properties, we adopted



Scheme 1. Schematic illustration of our strategy for protein labeling.

an intramolecular fluorophore displacement strategy, as has already been successfully applied to the fluorometric detection of nitric oxide,[11] pH,[12] and anions.[13] We hypothesized that if an intramolecular fluorescent ligand coordinates to the metal chelated by NTA with a relatively weak affinity, it would be displaced in the presence of the hexahistidine peptide sequence, resulting in enhanced fluorescence.

Design of fluorescent probes: The structures of "hemilabile" ligands consist of the fluorophore, a linker, and the metal–NTA complex as the His-tag recognition site (Figure 1).

Several linkers were employed because the nature of the linker is expected to influence the affinity of the fluorophore coordination to the metal–NTA complex. Hydroxycoumarin derivatives, such as calcein blue, are known indicators of various metal ions.[14] The fluorescence of such a molecule is significantly reduced upon coordination with a metal ion, and so we considered that hydroxycoumarin

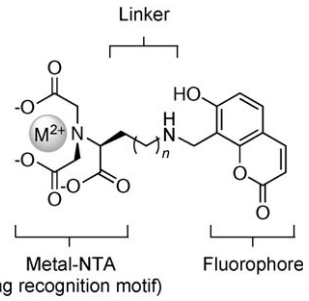
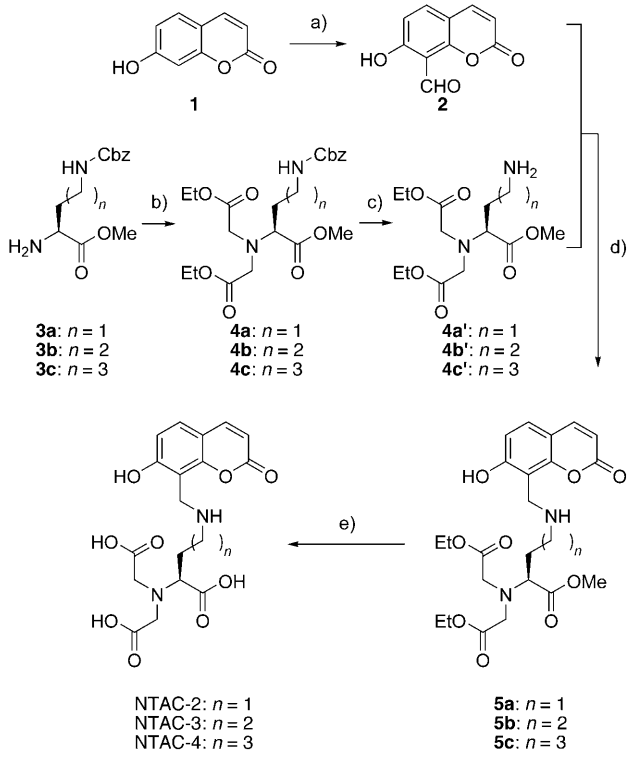


Figure 1. Structure of the designed fluorescent probes; M^{2+} is a metal ion that can be chelated stably by NTA. ($n = 1$–3).

would be a good candidate fluorophore for our purpose. The hydroxyl group of coumarin and/or the secondary amine may coordinate to the metal–NTA complex. To make the probes switchable, metal–NTA should quench the fluorescence of hydroxycoumarin efficiently. Therefore, we screened various metal ions from the standpoint of fluorescence quenching, as described below.

Synthesis of ligand molecules with fluorophore: The designed molecules were synthesized according to Scheme 2. Commercially available 7-hydroxycoumarin (**1**) was formylated selectively at the 8-position with paraformaldehyde and triethylamine.[15] The NTA units were synthesized from **3a–c**. Compound **3a** was synthesized by a Curtius reaction starting from *N*-α-*tert*-butoxycarbonyl-L-glutamic acid γ-benzyl ester according to the reported procedure.[16] Compounds **3b** and **3c** were readily synthesized from *N*-δ-benzyloxycarbonyl-L-ornithine and *N*-ε-benzyloxycarbonyl-L-lysine, respectively.[16,17] Bisalkylation of the α nitrogen of **3a–c** with ethyl bromoacetate in the presence of potassium carbonate provided the triesters **4a–c**. Deprotection of the benzyloxycarbonyl (Cbz) group by hydrogenolysis gave the corresponding primary amines, and then condensation with formylcoumarin **2**, followed by reduction afforded **5a–c**. Hydrolysis with lithium hydroxide provided the desired NTACs, which were purified by reverse-phase HPLC. This synthesis features reductive amination of an aldehyde and amine, which may be compatible with various other fluorophores and tag-peptide-recognizing motifs.



Scheme 2. Synthesis of NTACs: a) paraformaldehyde, Et_3N, CH_3CN, reflux, 10 h; 13%; b) ethyl bromoacetate, K_2CO_3, CH_3CN, reflux, 20 h; 56% (**4a**), 91% (**4b**), 76% (**4c**); c) 10% Pd/C, MeOH, RT, 12 h; d) 1) AcOH, THF or dichloroethane, RT, 2–6 h; 2) $NaBH_3CN$, THF, RT, 12 h; 37% (**5a**), 55% (**5b**), 43% (**5c**); e) LiOH (1 N), 30% aq MeOH, 0 °C, 2 h; 26% (NTAC-2), 45% (NTAC-3), 31% (NTAC-4).

Screening of metal ions: To find the appropriate metal ions for NTACs, the fluorescence change was investigated upon addition of various metal ions. Metal ions that can efficiently quench the fluorescence of the hydroxycoumarin fluorophore are required to develop "turn-on" fluorescent probes. All the NTACs were highly fluorescent in Tris buffer (50 mM, pH 7.4). Although the majority of the 21 metal ions examined did not exhibit efficient quenching of hydroxycoumarin fluorescence, several showed concentration-dependent quenching. Representative results are shown in Figure 2. Among the metal ions that we used, Ni^{2+} and Co^{2+} were found to be the best from the standpoint of fluorescence quenching, and they were selected for further study because both Ni^{2+}–NTA and Co^{2+}–NTA are known to interact selectively with hexahistidine sequences.

A Job's plot analysis was performed to determine the complexation stoichiometry of NTACs with Ni^{2+} or Co^{2+} in neutral aqueous buffer. Eleven solutions containing NTAC and Ni^{2+} or Co^{2+} in different ratios were prepared, and the total concentration was kept at 10 μM. Fluorescence changes (excitation wavelength: 365 nm, emission wavelength: 455 nm) of these samples were plotted as a function of the molar fraction of NTACs. The plots showed a peak at 0.5 (Figure 3), which indicated the formation of a 1:1 complex. This is consistent with our hypothesis that hydroxycoumarin coordinates intramolecularly to the metal, which is chelated by NTA.

Fluorescence response of Co^{2+}–NTAC and Ni^{2+}–NTAC with hexahistidine peptide: We investigated the fluorescence change of NTAC–metal complexes upon addition of a model tag peptide. We first prepared a solution of Ni^{2+}–NTAC or Co^{2+}–NTAC complex by mixing NTAC solution and $NiCl_2$ or $CoCl_2$ solution in Tris buffer (50 mM, pH 7.4). A model hexahistidine tag peptide **6** (H-$(His)_6$-Tyr-NH_2; Tyr was attached to allow convenient determination of concentration) was added to the Ni^{2+}–NTAC or Co^{2+}–NTAC solution, and the fluorescence spectra were obtained. Represen-

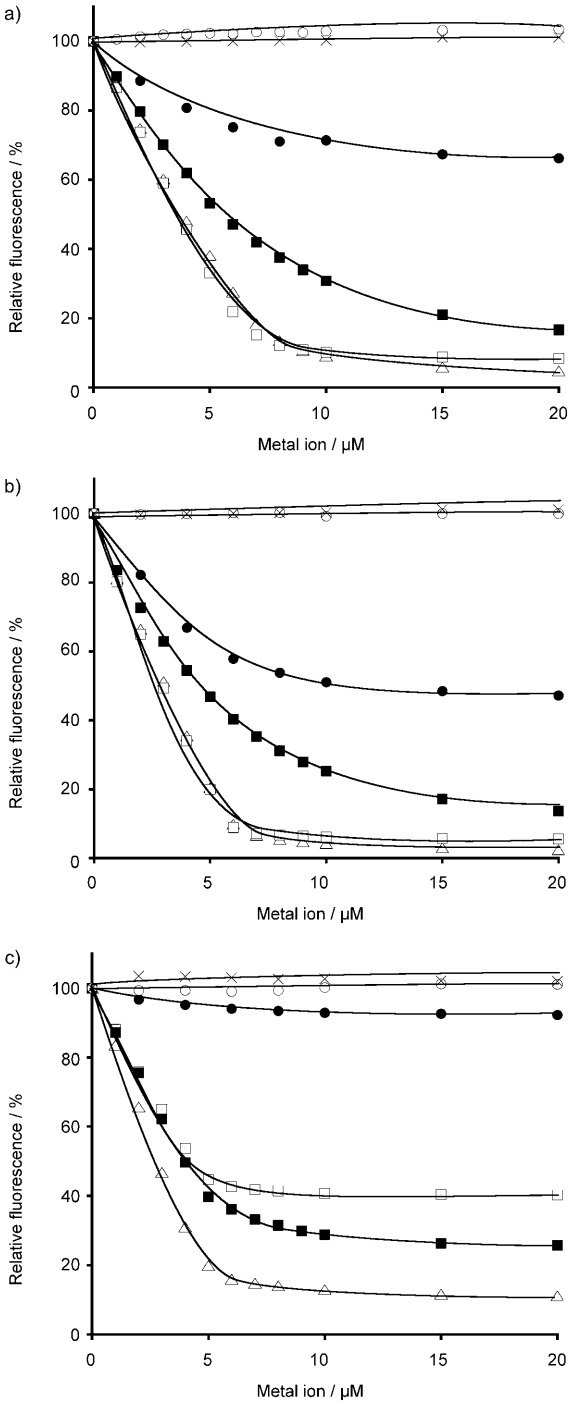


Figure 2. Relative fluorescence intensities of NTACs (5 μM) in the presence of various metal ions (Mn^{2+}, Co^{2+}, Ni^{2+}, Cu^{2+}, Ca^{2+}, Mg^{2+}) in Tris buffer (50 mM, pH 7.4). a) NTAC-2, b) NTAC-3, c) NTAC-4. Excitation: 365 nm, emission: 455 nm. ●: Mn^{2+}, △: Co^{2+}, □: Ni^{2+}, ■: Cu^{2+}, ○: Ca^{2+}, ×: Mg^{2+}.

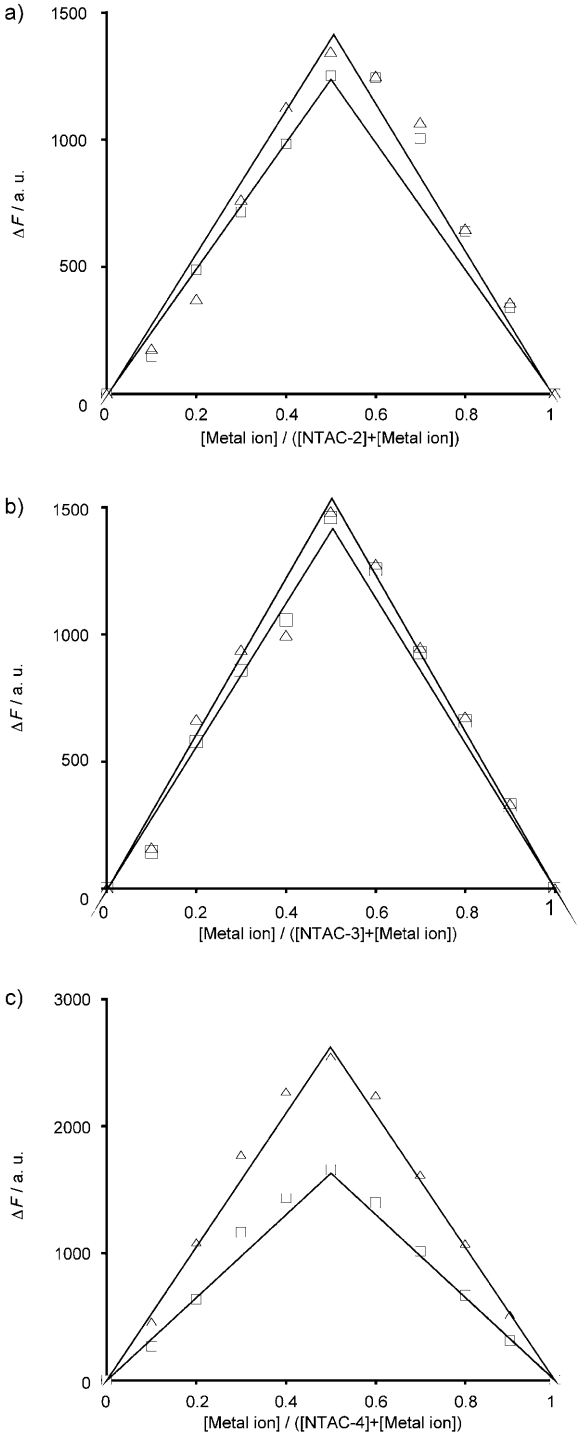


Figure 3. Job's plot of the fluorescence changes upon complexation of NTACs with Ni^{2+} or Co^{2+} in Tris buffer (50 mM, pH 7.4) at 25 °C. a) NTAC-2, b) NTAC-3, c) NTAC-4. [NTAC]+[metal ion] = 10 μM. Excitation: 365 nm, emission: 455 nm. □: Ni^{2+}, △: Co^{2+}.

tative results are shown in Figure 4a. The fluorescence increased in a concentration-dependent manner. All NTAC–metal complexes gave similar results. Most existing probes that comprise a fluorophore and a metal-ion-chelating NTA moiety contain Ni^{2+} as the metal ion;[8,10] however, Co^{2+} complexes showed larger fluorescence changes than Ni^{2+}–NTAC complexes in our case. In addition, Ni^{2+}–NTAC complexes needed a much longer time for equilibration (more than 3 h) compared with Co^{2+}–NTAC complexes (~10 min).

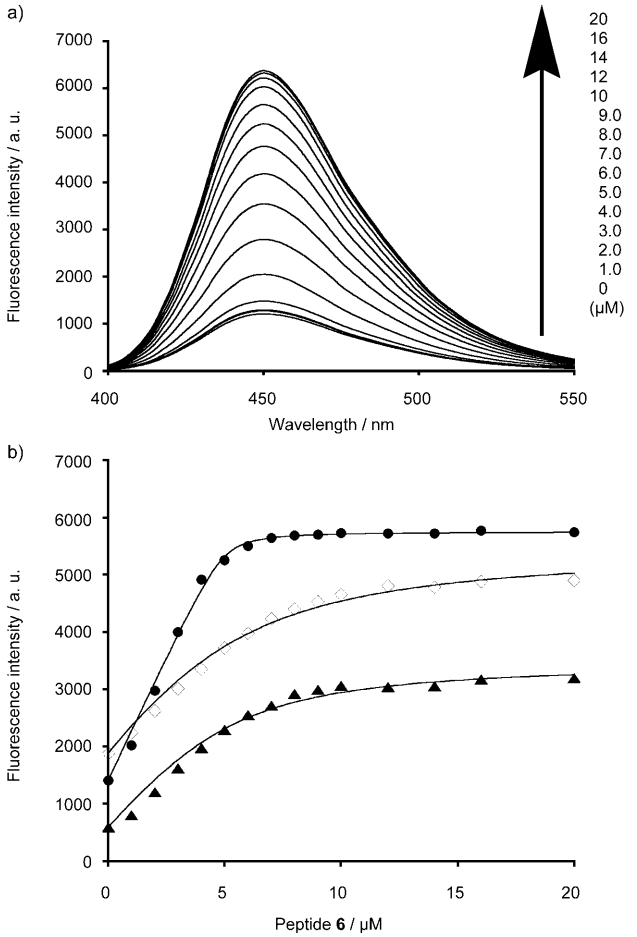


Figure 4. a) Emission spectra of Co^{2+}–NTAC-4 complexes in the presence of peptide **6** (0, 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 12, 14, 16, 20 μM) in Tris buffer (50 mM, pH 7.4). Co^{2+}–NTAC complex was prepared in situ by mixing NTAC and $CoCl_2$ (NTAC-4: 5 μM, $CoCl_2$: 10 μM). All spectra were obtained with excitation at 365 nm. b) Fluorescence response of Co^{2+}–NTAC complex (5 μM) in the presence of peptide **6** in Tris buffer (50 mM, pH 7.4). Co^{2+}–NTAC complex was prepared by mixing equimolar amounts of NTAC and $CoCl_2$ (NTAC: 5 μM, $CoCl_2$: 5 μM). Excitation: 365 nm, emission: 455 nm. ◇: Co^{2+}–NTAC-2, ▲: Co^{2+}–NTAC-3, ●: Co^{2+}–NTAC-4.

Thus, for our present purpose, Co^{2+}–NTAC complexes are superior. The fluorescence increase of Co^{2+}–NTACs was dependent on the peptide concentration, as shown in Figure 4b. The intrinsic fluorescence of the Co^{2+}–NTAC complex was very weak, whereas strong blue fluorescence was observed in the presence of peptide **6**, as shown in Figure S1 (see the Supporting Information). The final fluorescence recovery upon binding of Co^{2+}–NTAC complex to the peptide **6** reached >90% of the fluorescence intensity of the corresponding metal-free dye.

To determine the stoichiometry of the Co^{2+}–NTAC and hexahistidine peptide **6** complexes in solution, Job's plot analyses of the fluorometric changes were made. The Co^{2+}–NTAC complexes were generated in situ by combining NTAC with $CoCl_2$ in a 1:1 ratio in Tris buffer (50 mM, pH 7.4). Eleven solutions that contained Co^{2+}–NTAC and peptide **6** in different ratios were prepared, and the total concentration was kept at 10 μM. Job's plots showed a maximum at the molar ratio of 0.5, which indicated the formation of a 1:1 complex (Figure 5). The Job's plot for NTAC-4 at a lower ratio of peptide **6** showed nonlinearity. The reason for this observation is currently unclear.

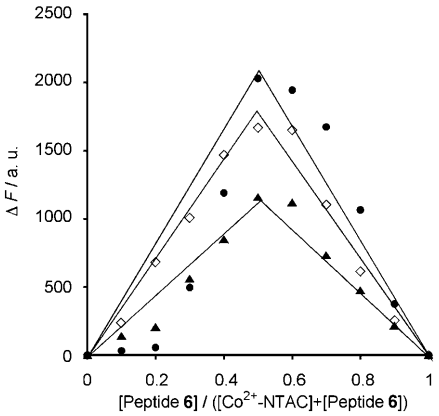


Figure 5. Job's plot of the fluorescence changes upon complexation of Co^{2+}–NTAC complexes with peptide **6** in 50 mM Tris buffer (pH 7.4) at 25 °C. [Co^{2+}–NTAC complexes]+[peptide **6**] = 10 μM. Co^{2+}–NTAC complexes were prepared by mixing equimolar amounts of NTACs and $CoCl_2$. Excitation: 365 nm, emission: 455 nm. ◇: Co^{2+}–NTAC-2, ▲: Co^{2+}–NTAC-3, ●: Co^{2+}–NTAC-4.

Fluorescence and chemical properties of Co^{2+}–NTAC complexes: The fluorescence and chemical properties of Co^{2+}–NTAC complexes are summarized in Table 1. The Co^{2+}–NTAC complexes were generated in situ by combining NTAC with $CoCl_2$ in a 1:10 ratio to suppress the NTAC fluorescence completely. The fluorescence spectra were measured in Tris buffer (50 mM, pH 7.4) in the absence or presence of peptide **6**. The fluorescence quantum yields were 0.033–0.10 before addition of peptide **6**, and were increased 7.9–22-fold to 0.67–0.79 by the addition of peptide **6**. Further, the binding affinity for peptide **6** was assessed from the fluorescence intensity. In this case, Co^{2+}–NTAC complexes were generated by combining NTAC with $CoCl_2$ in a 1:1 ratio, assuming that formation of NTAC–Co^{2+} complex is complete, since the interaction of NTAC with Co^{2+} is very strong. The apparent dissociation constants (K_d) of Co^{2+}–NTAC-2, Co^{2+}–NTAC-3, and Co^{2+}–NTAC-4 for peptide **6** were calculated to be 2.2×10^{-6}, 1.4×10^{-6}, and $5.7\times$

Table 1. Chemical properties of Co^{2+}–NTAC.[a]

Compound	Fluorescence quantum yield[b]		K_d [M][c]
	Co^{2+}–NTAC	Co^{2+}–NTAC–peptide **6**	
NTAC-2	0.073	0.73	$(2.2\pm0.4)\times10^{-6}$
NTAC-3	0.033	0.67	$(1.4\pm0.4)\times10^{-6}$
NTAC-4	0.10	0.79	$(5.7\pm2.7)\times10^{-8}$

[a] All data were measured in Tris buffer (50 mM, pH 7.4). [b] Quantum yields were calculated by using that of quinine sulfate in H_2SO_4 (1.0 N) as a standard.[19] [c] Dissociation constants of Co^{2+}–NTAC complexes for peptide **6**.

10^{-8} M, respectively. These K_d values are comparable with those of other Ni^{2+}–NTA based probes (1-20 μM),[8a,b,d] and suggest that Co^{2+}–NTAC-4 would be the most efficient among the molecules that we have developed. The long spacer would favor easy displacement of the fluorophore by the peptide.

Selectivity of Co^{2+}–NTAC-4: To confirm the selectivity of the fluorescence response of Co^{2+}–NTAC-4, the fluorescence spectra were measured in the presence of angiotensin I (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH). Although this peptide contains two histidines, they are not sequential. As shown in Figure 6, no fluorescence change of

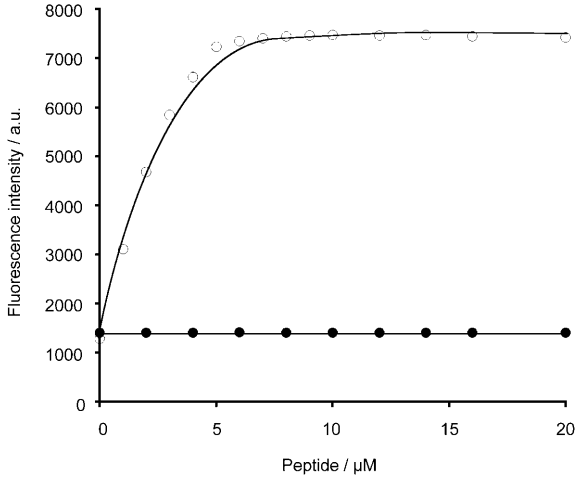


Figure 6. Fluorescence response of Co^{2+}–NTAC-4 complex (5 μM) in the presence of angiotensin I (H-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-OH) or H_6-angiotensin I (H-$(His)_6$-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-NH_2) in Tris buffer (50 mM, pH 7.4). Co^{2+}–NTAC complex was prepared by mixing equimolar amounts of NTAC and $CoCl_2$ (NTAC-4: 5 μM, $CoCl_2$: 5 μM). Excitation: 365 nm, emission: 455 nm. ○: H_6-angiotensin I, ●: angiotensin I.

Co^{2+}–NTAC-4 complex was observed upon addition of angiotensin I. On the other hand, an analogous peptide that included the hexahistidine sequence, H_6-angiotensin I (H-$(His)_6$-Asp-Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu-NH_2), showed strong fluorescence enhancement. These results suggest that our molecules selectively recognize polyhisitidine sequences.

Conclusions

We have developed novel "turn-on" fluorescent probes that bind selectively to a hexahistidine tag peptide via a metal ion–NTA moiety, with a substantial enhancement of their fluorescence. The new probes consist of a hydroxycoumarin fluorophore that is joined via a linker moiety to Co^{2+}–NTA. These complexes are intrinsically only weakly fluorescent, probably due to the intramolecular coordination of the fluorophore by the metal, but displacement of the fluorophore with peptide **6** in pH 7.4 buffered aqueous solutions produced a selective increase in the fluorescence quantum yield of up to 22-fold. No enhancement was observed upon addition of angiotensin I, which contains two nonadjacent histidine residues. The strategy described here should be applicable to various other fluorophores/functional compounds. Because the hexahistidine tag is the most frequently applied affinity tag in recombinant protein expression, numerous applications for selective fluorescent labeling or functionalization of recombinant proteins can be envisioned. Further work is in progress in our laboratory, especially on protein labeling.

Experimental Section

General information: All reagents and solvents were of the highest commercial quality and were used without further purification unless otherwise noted. THF and diethyl ether (Et_2O) were distilled from Na/benzophenoneketyl immediately prior to use. CH_2Cl_2 was distilled from CaH_2 immediately prior to use. MeOH and EtOH were distilled from Mg/I_2. TLC was performed on Merck precoated plates (silica gel 60 F254, 0.25 mm), and bands were visualized by fluorescence quenching. TLC plates were also stained with potassium permanganate. ^{1}H NMR spectra were recorded on a JEOL GSX-400 or a JEOL JNM-LA 500 at 400 or 500 MHz, respectively. ^{13}C NMR spectra were recorded on either a JEOL JNM-LA 500 or a Bruker AVANCE 600 at 125 or 150 MHz, respectively. Chemical shifts are expressed as parts per million (ppm) by using solvent as an internal standard. IR spectra were recorded on a JASCO FT/IR-680 FTIR spectrophotometer. Fast atom bombardment mass spectra (FABMS) were measured with a JEOL JMS-LCMATE mass spectrometer, by using 3-nitrobenzyl alcohol as the matrix. Matrix-assisted laser desorption ionization time of flight mass spectrometry (MALDI-TOF MS) was done with a Shimadzu AXIMA-CFR-NC by using α-cyano-4-hydroxycinnamic acid as the matrix. HRMS data were obtained with a JEOL JMS-SX102A or a Bruker Daltonics APEX-III mass spectrometer. Column chromatography was performed on BW-200 or -300 (Fuji Silysia Chemical, Aichi, Japan). Optical rotations were measured on a JASCO DIP-1000 polarimeter at room temperature, by using the sodium D line.

8-Formyl-7-hydroxycoumarin (2): Paraformaldehyde (448 mg, 14 mmol) and Et_3N (1.1 mL, 8.0 mmol) were added to a solution of 7-hydroxycoumarin (**1**; 324 mg, 2.0 mmol) in CH_3CN (10 mL). The mixture was refluxed for 10 h. The solution was cooled to room temperature and neutralized with HCl (1 N). The mixture was extracted with CH_2Cl_2 (10 mL × 3), washed with brine (10 mL × 3), and dried over $MgSO_4$. Purification by flash column chromatography on silica gel (EtOAc/*n*-hexane, 1:3) afforded **2** (50.5 mg, 13%) as a colorless solid. ^{1}H NMR (400 MHz, $CDCl_3$): δ = 12.24 (s, 1H), 10.61 (s, 1H), 7.67 (d, J = 9.6 Hz, 1H), 7.61 (d, J = 8.8 Hz, 1H), 6.90 (d, J = 8.8 Hz, 1H), 6.34 ppm (d, J = 9.6 Hz, 1H); MS (FAB): m/z: 191 $[M+H]^+$. These data were consistent with the literature data for this compound.[15]

General procedure for *N*-alkylation: Ethyl bromoacetate (10 equiv), followed by finely ground K_2CO_3 (10–20 equiv) were added to a solution of an appropriate side-chain-protected amino acid methyl ester (1 equiv) in CH_3CN. The reaction was heated at reflux for 20 h, cooled to room temperature, and concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel.

Methyl (2*S*)-4-*N*-Benzyloxycarbonyl-2-*N*-bis(2-ethoxy-2-oxoethyl)-2,4-diaminobutanoate (4a): According to the general procedure, a solution of **3a**[16] (3.97 g, 14.9 mmol) in CH_3CN (60 mL) was treated with ethyl bromoacetate (16.5 mL, 150 mmol) and K_2CO_3 (20.6 g, 150 mmol). The crude product was purified by column chromatography on silica gel (EtOAc/*n*-hexane 1:4) to afford **4a** (3.63 g, 56%) as a pale-yellow oil. R_f = 0.48 (EtOAc/*n*-hexane 2:3); $[\alpha]_D^{20} = -41.6$ (c = 1.05 in EtOH);

^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.38–7.29 (m, 5H), 6.44 (brs, 1H), 5.10 (m, 2H), 4.13 (q, J = 7.2 Hz, 4H), 3.68 (s, 3H), 3.57 (m, 4H), 3.58–3.54 (s, 1H), 3.45–3.39 (m, 2H), 2.02–1.95 (m, 1H), 1.82–1.74 (m, 1H), 1.22 ppm (t, J = 7.2 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 172.5, 171.1, 156.5, 136.9, 128.1, 127.6, 127.5, 66.0, 61.9, 60.6, 52.5, 51.3, 37.5, 29.1, 13.9 ppm; IR (film): $\tilde{\nu}$ = 3366, 2954, 1733, 1520 cm^{-1}; HRMS (EI;70 eV): m/z: calcd for $C_{21}H_{30}N_2O_8$: 438.2002 $[M]^+$; found: 438.2002.

***N*-δ-Benzyloxycarbonyl-*N*-α-bis(2-ethoxy-2-oxoethyl)-L-ornithine methyl ester (4b)**: According to the general procedure, a solution of **3b**[17] (2.54 g, 9.08 mmol) in CH_3CN (50 mL) was treated with ethyl bromoacetate (10.1 mL, 90.8 mmol) and K_2CO_3 (25.1 g, 181 mmol). The crude product was purified by column chromatography on silica gel (EtOAc/*n*-hexane 2:3) to afford **4b** (3.72 g, 91%) as a pale-yellow oil. R_f = 0.30 (EtOAc/*n*-hexane, 2:3); $[\alpha]_D^{20}$ = −21.3 (c = 1.05 in EtOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.34–7.28 (m, 5H), 5.08 (brs, 2H), 4.12 (q, J = 7.3 Hz, 4H), 3.67 (s, 3H), 3.61 (m, 4H), 3.62 (s, 3H), 3.43 (m, 1H), 3.24–3.23 (m, 2H), 1.74–1.68 (m, 4H), 1.33–1.22 ppm (m, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 172.3, 170.8, 156.0, 136.3, 127.9, 127.4, 127.4, 65.8, 63.9, 60.1, 52.2, 50.9, 40.1, 27.0, 25.7, 13.7 ppm; IR (film): $\tilde{\nu}$ = 3383, 2953, 1730, 1537 cm^{-1}; HRMS (EI; 70 eV): m/z: calcd for $C_{22}H_{32}N_2O_8$: 452.2159 $[M]^+$; found: 452.2151.

***N*-ε-Benzyloxycarbonyl-*N*-α-bis(2-ethoxy-2-oxoethyl)-L-lysine methyl ester (4c)**: This compound was synthesized according to the reported procedure (yield: 73%). The analytical data were consistent with the literature.[18]

General procedure for coupling to the hydroxycoumarin derivative: To a solution of an appropriate triester (0.85–1.5 equiv) in CH_3OH was added 10% Pd/C. This mixture was stirred vigorously under H_2 (1 atm) for 12 h at room temperature. The mixture was filtered through Celite, and the filtrate was concentrated under reduced pressure. The resulting residue was dissolved in THF or dichloroethane, and a solution of **2** (1 equiv) and acetic acid (2 equiv) in THF or dichloroethane was added. The mixture was stirred for 2–6 h at room temperature, then sodium cyanoborohydride (1.5–2 equiv) was added. Stirring was continued for an additional 12 h at room temperature, the mixture was chilled to 0°C, and water was added with stirring. The solution was concentrated under reduced pressure. The crude product was purified by flash column chromatography on silica gel.

***N*-[3-(Bisethoxycarbonylmethylamino)-3-ethoxycarbonylpropyl]-7-hydroxy-8-aminomethylcoumarin (5a)**: According to the general procedure, **4a** (137 mg, 0.45 mmol) was treated in CH_3OH (10 mL) with 10% Pd/C (15 mg) under H_2. After work-up, the resulting residue **4a′** was dissolved in THF (2.0 mL) and a solution of **2** (100 mg, 0.53 mmol) and acetic acid (60.2 mL, 1.05 mmol) in THF (2.0 mL) was added. The mixture was stirred for 6 h, then sodium cyanoborohydride (66.1 mg, 1.05 mmol) was added. The crude product was purified by column chromatography on silica gel (MeOH/CH_2Cl_2 1:19) to afford **5a** (79.7 mg, 37%) as a pale-yellow oil. R_f = 0.23 (MeOH/CH_2Cl_2 1:9); $[\alpha]_D^{20}$ = −10.22 (c = 1.05 in EtOH); ^{1}H NMR (500 MHz, $CDCl_3$): δ = 7.65 (d, J = 9.5 Hz, 1H), 7.38 (d, J = 8.6 Hz, 1H), 6.99 (d, J = 8.6 Hz, 1H), 6.21 (d, J = 9.5 Hz, 1H), 4.71 (d, J = 12.7 Hz, 1H), 4.39 (d, J = 12.7 Hz, 1H), 4.11(q, J=7.1 Hz, 4H), 3.74 (s, 3H), 3.70–3.56 (m, 4H), 3.49–3.38 (m, 2H), 3.28–3.24 (m, 1H), 2.52–2.42 (m, 1H), 2.09–2.04 (m, 1H), 1.21 ppm (t, J = 7.1 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 171.8, 170.5, 160.9, 160.8, 154.1, 144.1, 129.9, 116.6, 112.2, 112.1, 108.4, 64.0, 61.8, 52.2, 47.7, 40.7, 24.5, 13.9 ppm; IR (film): $\tilde{\nu}$ = 2985, 1735, 1608, 1578 cm^{-1}; MS (FAB): m/z: 479 $[M+H]^+$; HRMS (ESI): m/z: calcd for $C_{21}H_{31}N_2O_8$: 479.2024 $[M+H]^+$; found: 479.2027.

8-*N*-[4-(Bisethoxycarbonylmethylamino)-4-ethoxycarbonylbutyl]aminomethyl-7-hydroxycoumarin (5b): According to the general procedure, **4b** (2.16 g, 6.78 mmol) was treated in CH_3OH (100 mL) with 10% Pd/C (200 mg) under H_2. After workup, the resulting residue **4b′** was dissolved in dichloroethane (20 mL) and a solution of **2** (860 mg, 4.52 mmol) and acetic acid (518 mL, 9.04 mmol) in dichloroethane (20 mL) was added. The mixture was stirred for 6 h, then sodium cyanoborohydride (426 mg, 6.78 mmol) was added. The crude product was purified by flash column chromatography on silica gel (MeOH/CH_2Cl_2 1:19) to afford **5b** (1.23 g, 55%) as a pale-yellow oil. R_f = 0.37 (MeOH/CH_2Cl_2 1:9); $[\alpha]_D^{20}$ = −18.3 (c = 1.05 in EtOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.69 (d, J = 9.5 Hz, 1H), 7.38 (d, J = 8.8 Hz, 1H), 6.99 (d, J = 8.8 Hz, 1H), 6.16 (d, J = 9.5 Hz, 1H), 4.54 (d, J = 13.7 Hz, 1H), 4.44 (d, J = 13.7 Hz, 1H), 4.15 (q, J = 7.1 Hz, 4H), 3.72 (s, 3H), 3.68–3.59 (m, 4H), 3.55–3.51 (m, 1H), 3.45–3.14 (m, 2H), 2.06–2.00 (m, 3H), 1.77–1.75 (m, 1H), 1.25 ppm (t, J = 7.1 Hz, 6H); ^{13}C NMR (100 MHz, $CDCl_3$): δ = 171.8, 171.6, 161.9, 160.8, 153.7, 144.4, 130.0, 111.0, 110.9, 104.8, 64.5, 61.5, 53.8, 51.9, 47.2, 41.2, 27.7, 24.2, 14.0 ppm; IR (film): $\tilde{\nu}$ = 2983, 1738, 1610, 1582 cm^{-1}; MS (FAB): m/z: 493 $[M+H]^+$; HRMS (ESI): m/z: calcd for $C_{23}H_{31}N_2O_9$: 493.2181 $[M+H]^+$; found: 493.2180.

8-*N*-[5-(Bisethoxycarbonylmethylamino)-5-ethoxycarbonylpentyl]aminomethyl-7-hydroxycoumarin (5c): According to the general procedure, **4c** (1.55 g, 4.65 mmol) was treated in CH_3OH (500 mL) with 10% Pd/C (150 mg) under H_2. After workup, the resulting residue **4c′** was dissolved in THF (10 mL) and a solution of **2** (590 mg, 3.10 mmol) and acetic acid (355 mL, 6.20 mmol) in THF (10 mL) was added. The mixture was stirred for 2 h, then sodium cyanoborohydride (214 mg, 4.65 mmol) was added. The crude product was purified by flash column chromatography on silica gel (MeOH/CH_2Cl_2 1:19) to afford **5c** (667 mg, 43%) as a pale-yellow oil. R_f = 0.33 (MeOH/CH_2Cl_2 1:9); $[\alpha]_D^{20}$ = −25.3 (c = 1.05 in EtOH); ^{1}H NMR (400 MHz, $CDCl_3$): δ = 7.62 (d, J = 9.5 Hz, 1H), 7.28 (d, J = 8.5 Hz, 1H), 6.80 (d, J = 8.5 Hz, 1H), 6.16 (d, J = 9.5 Hz, 1H), 4.35 (s, 2H), 4.14 (q, J = 7.1 Hz, 4H), 3.69 (s, 3H), 3.63–3.59 (m, 4H), 3.46–3.36 (m, 1H), 2.84–2.79 (m, 2H), 1.76–1.45 (m, 6H), 1.25 ppm (t, J = 7.1 Hz, 6H); ^{13}C NMR (125 MHz, $CDCl_3$): δ = 173.0, 172.1, 171.7, 163.6, 161.3, 153.2, 144.4, 128.5, 114.3, 111.1, 110.9, 107.6, 64.0, 60.9, 52.7, 52.6, 51.5, 48.3, 44.0, 29.6, 27.5, 22.9, 14.2 ppm; IR (film): $\tilde{\nu}$ = 2952, 1733, 1605, 1589 cm^{-1}; MS (FAB): m/z: 507 $[M+H]^+$; HRMS (ESI): m/z: calcd for $C_{25}H_{35}N_2O_8$: 507.2337 $[M+H]^+$; found: 507.2335.

General procedure for hydrolysis: A solution of LiOH (1N, 6 equiv) was added to a solution of an appropriate coumarin-containing triester (1 equiv) in 30% aqueous CH_3OH at 0°C. The reaction was allowed to warm to room temperature, stirred for 2 h, neutralized with 30% acetic acid, and concentrated under reduced pressure. The crude product was purified by reverse-phase HPLC.

***N*-[3-(Biscarboxymethylamino)-3-carboxypropyl]-7-hydroxy-8-aminomethylcoumarin (NTAC-2)**: According to the general procedure, LiOH (1N, 1.4 mL, 1.4 mmol) was added to a solution of **5a** (113 mg, 0.23 mmol) in 30% aqueous CH_3OH (10 mL). The crude product was purified by reverse-phase HPLC by using a linear gradient system from 18:82 to 20:80 CH_3CN/H_2O (0.1% TFA) over 20 min, and the collected fractions were lyophilized to afford NTAC-2 (32.6 mg, 26%) as a white solid. The purity of this compound was determined as >97% by HPLC analysis. ^{1}H NMR (500 MHz, DMSO): δ = 7.90 (d, J = 9.5 Hz, 1H), 7.51 (d, J = 8.6 Hz, 1H), 6.91 (d, J = 8.6 Hz, 1H), 6.26 (d, J = 9.5 Hz, 1H), 4.96 (d, J = 14.3 Hz, 1H), 4.83 (d, J = 14.3 Hz, 1H), 4.11–4.07 (m, 1H), 4.04–3.96 (m, 4H), 3.37–3.34 (m, 2H), 2.32–1.86 ppm (m, 2H); ^{13}C NMR (125 MHz, DMSO): δ = 173.0, 172.8, 160.0, 159.8, 153.9, 144.7, 129.0, 112.7, 111.3, 111.3, 108.8, 62.9, 53.2, 43.0, 34.8, 23.4 ppm; IR (KBr): $\tilde{\nu}$ = 3092, 1721, 1688, 1612, 1579 cm^{-1}; MS (MALDI): m/z: 409.5 $[M+H]^+$; HRMS (FAB): m/z: calcd for $C_{18}H_{17}N_2O_8$: 389.0985 $[M-H_2O-H]^-$; found: 389.0994.

8-*N*-[4-(Biscarboxymethylamino)-4-carboxylbutyl]aminomethyl-7-hydroxycoumarin (NTAC-3): According to the general procedure, LiOH (1N 1.2 mL, 1.2 mmol) was added to a solution of **5b** (100 mg, 0.20 mmol) in 30% aqueous CH_3OH (10 mL). The crude product was purified by reverse-phase HPLC by using a linear gradient system from 15:85 to 17:83 CH_3CN/H_2O (0.1% TFA) over 20 min, and the collected fractions were lyophilized to dryness to afford NTAC-3 (49.0 mg, 45%) as a white solid. The purity of this compound was determined to be >96% by HPLC analysis. ^{1}H NMR (500 MHz, CD_3OD): δ = 7.90 (d, J = 9.5 Hz, 1H), 7.51 (d, J = 8.6 Hz, 1H), 6.91 (d, J = 8.6 Hz, 1H), 6.26 (d, J = 9.5 Hz, 1H), 4.96 (d, J = 14.3 Hz, 1H), 4.83 (d, J = 14.3 Hz, 1H), 4.04–3.96 (m, 4H), 4.11–4.07 (m, 1H), 3.37–3.34 (m, 2H), 2.32–1.86 ppm (m, 4H); ^{13}C NMR (125 MHz, CD_3OD): δ = 162.9, 161.7, 155.7, 146.3, 130.5, 114.6, 113.3, 112.5, 110.6, 65.6, 55.4, 48.2, 40.8, 24.2, 21.8 ppm; IR (KBr): $\tilde{\nu}$ = 2952, 1717, 1651, 1608, 1578 cm^{-1}; MS (MALDI): m/z: 423.2 $[M+H]^+$; HRMS

(FAB): *m/z*: calcd for $C_{19}H_{19}N_2O_8$: 403.1141 $[M-H_2O-H]^-$; found: 403.1130.

8-*N*-[5-(Biscarboxymethylamino)-5-carboxylpentyl]aminomethyl-7-hydroxycoumarin (NTAC-4): According to the general procedure, LiOH (1 N, 1.2 mL, 1.2 mmol) was added to a solution of **5c** (100 mg, 0.20 mmol) in 30% aqueous CH_3OH (10 mL). The residue was purified by reverse-phase HPLC by using a linear gradient system from 13:87 to 15:85 CH_3CN/H_2O (0.1% TFA) over 20 min, and the collected fractions were lyophilized to afford NTAC-4 (33.8 mg, 31%) as a white solid. The purity of this compound was determined as >92% by HPLC analysis. ^{1}H NMR (400 MHz, CD_3OD): $\delta=7.69$ (d, $J=9.2$ Hz, 1H), 7.26 (d, $J=8.6$ Hz, 1H), 6.57 (d, $J=8.6$ Hz, 1H), 5.90 (d, $J=9.2$ Hz, 1H), 4.21 (s, 2H), 3.53 (s, 4H), 3.39–3.35 (m, 1H), 2.94–2.90 (m, 2H), 1.68–1.33 ppm (m, 6H); ^{13}C NMR (150 MHz, DMSO): $\delta=174.2$, 173.9, 160.3, 159.7, 153.9, 144.7, 130.4, 112.5, 111.4, 111.1, 105.5, 64.8, 54.6, 46.9, 38.4, 29.2, 24.9 ppm; IR (KBr): $\tilde{\nu}=3030$, 1717, 1684, 1612, 1580 cm^{-1}; HRMS (FAB): *m/z*: calcd for $C_{20}H_{23}N_2O_9$: 435.14033 $[M-H]^-$; found: 435.14127.

Synthesis of peptides: Angiotensin I was purchased from the Peptide Institute (Osaka, Japan). H-His_6-Tyr-NH_2 (**6**) and H_6-angiotensin I were synthesized on TentaGel S RAM resin (0.36 mmol g^{-1} Watanabe Chemical Industries, Hiroshima, Japan). Four equivalents of *N*-9-fluorenylmethyloxycarbonyl (Fmoc) amino acid, 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium hexafluorophosphate (HBTU), *N*-hydroxybenzotriazole hydrate (HOBt), and diisopropylethylamine (DIPEA) (8 equiv) were used for each coupling. DMF was the solvent. For deprotection steps, 20% piperidine in DMF was used. The completion of amino acid couplings was monitored with the Kaiser test. The peptide was cleaved and deprotected by shaking with TFA/triisopropylsilane/H_2O (95:2.5:2.5 v/v/v) for 2 h, followed by precipitation into cold diethyl ether. The precipitate was collected by centrifugation/decantation prior to purification. The purification was performed by reverse-phase HPLC by using a linear gradient system from 11:89 to 13:87 CH_3CN/H_2O (0.1% TFA) over 20 min (peptide **6**) and 20:80 to 22:78 CH_3CN/H_2O (0.1% TFA) over 20 min (H_6-angiotensin I), and the collected fractions were lyophilized to afford a white solid. The molecular weights of the peptides were confirmed by mass spectrometry. MS (MALDI): *m/z*: calcd for $C_{45}H_{55}O_{20}N_8$: 1003.5 $[M+H]^+$; found: 1003.5 (peptide **6**); calcd for $C_{98}H_{133}O_{36}N_{19}$: 2118.1 $[M+H]^+$; found: 2118.0 (H_6-angiotensin I).

Fluorometric analysis: Fluorescence spectra were measured with a Hitachi F4500. The slit width was 5.0 nm for excitation and emission. The photomultiplier voltage was 700 V. Relative quantum yields of fluorescence were obtained by comparing the area under the corrected emission spectrum of the test sample at 366 nm excitation with that of a solution of quinine sulfate in H_2SO_4 (1.0 N) (quantum yield: 0.55).[19] $CoCl_2$ (10 equiv to NTACs) and peptide **1** (50 equiv to NTACs) were used for the determination of quantum yields.

Determination of apparent dissociation constants: Aliquots of a stock solution of peptide **6** were added to a cuvette that contained a solution of Co^{2+}–NTAC complex (3.0 mL, 5 μM) in Tris buffer (50 mM, pH 7.4). To determine the dissociation constant (K_d), the change of fluorescence intensity with increasing peptide **6** concentration was measured. Excitation and emission wavelengths were set at 355 and 455 nm, respectively. The value of K_d was obtained by nonlinear least-squares fitting to Equation (1),[20] since a 1:1 complex is formed between Co^{2+}–NTAC complex and peptide **6** as shown from the results of a Job's plot. The K_d value was calculated by using a Kaleida Graph (Synergy Software, Reading, PA, USA).

$$\frac{(F-F_{min})}{(F_{max}-F_{min})} = \frac{\left[([MC]_0+[P]_0+K_d)-\{([MC]_0+[P]_0+K_d)^2-4[MC]_0[P]_0\}^{1/2}\right]}{[2\,MC]_0} \quad (1)$$

in which F is the observed fluorescence intensity, F_{min} is the minimum fluorescence intensity (in the absence of peptide **6**), and F_{max} is the maximum fluorescence intensity. $[MC]_0$ and $[P]_0$ are the total concentrations of Co^{2+}–NTAC complex and peptide **6**, respectively.

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