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Modulation of Luminescence Intensity of Lanthanide Complexes by Photoinduced Electron Transfer and Its Application to a Long-Lived Protease Probe

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Abstract: Luminescent lanthanide complexes (Tb^{3+}, Eu^{3+}, etc.) have excellent properties for biological applications, including extraordinarily long lifetimes and large Stokes shifts. However, there have been few reports of lanthanide-based functional probes, because of the difficulty in designing suitable complexes with a luminescent on/off switch. Here, we have synthesized a series of complexes which consist of three moieties: a lanthanide chelate, an antenna, and a luminescence off/on switch. The antenna is an aromatic ring which absorbs light and transmits its energy to the metal, and the switch is a benzene derivative with a different HOMO level. If the HOMO level is higher than a certain threshold, the complex emits no luminescence at all, which indicates that the lanthanide luminescence can be modulated by photoinduced electron transfer (PeT) from the switch to the sensitizer. This approach to control lanthanide luminescence makes possible the rational design of functional lanthanide complexes, in which the luminescence property is altered by a biological reaction. To exemplify the utility of our approach to the design of lanthanide complexes with a switch, we have developed a novel protease probe, which undergoes a significant change in luminescence intensity upon enzymatic cleavage of the substrate peptide. This probe, combined with time-resolved measurements, was confirmed in model experiments to be useful for the screening of inhibitors, as well as for clinical diagnosis.

Introduction

Sensitive detection of compounds of interest is one of the greatest challenges not only in analytical chemistry but also in biology and medicine. For decades, scientists have been developing a wide range of sophisticated spectroscopic approaches to address this issue. Fluorescence is one of the most widely used techniques in this field, because it offers many advantages, including sensitivity, simplicity, and robustness. From the early 1980s,[1] functional fluorescence probes have been developed, which change their fluorescence properties in response to their reaction with the chemical species of interest. Since they make possible a sensitive, safe, rapid, quantitative, and facile detection of the species both in vitro and in vivo, considerable attention has been devoted to the development of such probes, the targets of which include Ca^{2+},[1] Zn^{2+},[2] NO,[3] and enzymes.[4] These probes, however, have common disadvantages involving background fluorescence and scattered light. In the physiological environments such as cytoplasm and serum, sources of background fluorescence are present everywhere. Light scattering can also be a difficult problem when a conventional fluorescence probe, whose Stokes shift is only 20−30 nm, is used. These factors considerably impair the quality of measurements, especially when the scale of the assay is small or the probe concentration is low.

Luminescent lanthanide complexes[5] provide an attractive solution to these problems. If an appropriate chelator is used, a lanthanide (especially Tb^{3+} or Eu^{3+}) complex has an extraordinarily long luminescence lifetime of the order of milliseconds, in contrast to a typical organic compound, which has a short

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(1) (a) Grynkiewicz, G.; Poenie, M.; Tsien, R. Y. *J. Biol. Chem.* **1985**, *260*, 3440−3450.
(2) Minta, A.; Kao, J. P. Y.; Tsien, R. Y. *J. Biol. Chem.* **1989**, *264*, 8171−8178.
(3) (a) Komatsu, K.; Kikuchi, K.; Kojima, H.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 10197−10204. (b) Gee, K. R.; Zhou, Z. L.; Qian, W. J.; Kennedy, R. *J. Am. Chem. Soc.* **2002**, *124*, 776−778.
(4) Kojima, H.; Nakatsubo, N.; Kikuchi, K.; Kawahara, S.; Kirino, Y.; Nagoshi, H.; Hirata, Y.; Nagano, T. *Anal. Chem.* **1998**, *70*, 2446−2453.
(5) Mizukami, S.; Kikuchi, K.; Higuchi, T.; Urano, Y.; Mashima, T.; Tsuruo, T.; Nagano, T. *FEBS Lett.* **1999**, *453*, 356−360.

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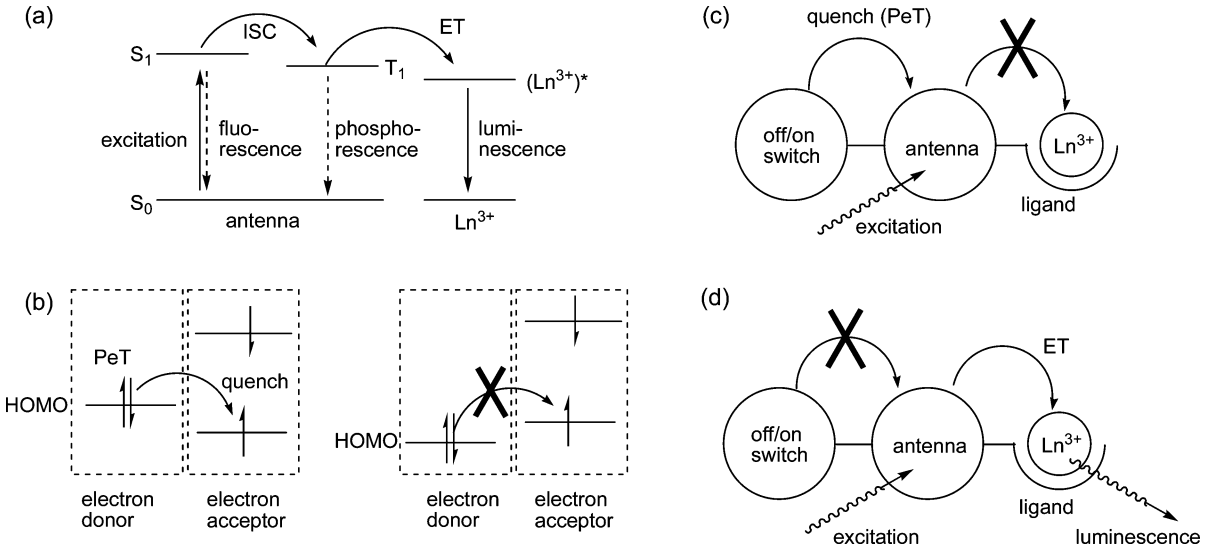

Figure 1. Schematic representations of our strategy to control the luminescence of lanthanide complexes. (a) Energy scheme for the luminescence process in a typical Ln^{3+} complex. (b) Schematic representation of the relationship between the HOMO energy level and photoinduced electron transfer. (c and d) Ln^{3+} complexes with a luminescence off/on switch. (c) Switch "off" model where PeT occurs, resulting in no Ln^{3+} luminescence. (d) Switch "on" model where PeT does not occur, resulting in strong Ln^{3+} luminescence.

fluorescence lifetime in the nanosecond region. Taking advantage of this feature, the influence of short-lived background fluorescence and scattered light can be reduced to a negligible level by the method termed time-resolved fluorescence (TRF) measurement.[6] In TRF measurement, the fluorescence signal is collected for a certain gate time after an appropriate delay time, following a pulsed excitation. By employing this method, it is readily possible to distinguish long-lived lanthanide luminescence from other contaminating signals. Furthermore, lanthanide complexes have large Stokes shifts (>200 nm), which also helps to reduce the background. Luminescent lanthanide complexes have been exploited as luminescent tags for TRF measurements in various fields,[7] especially immunoassays[8] and high throughput screenings,[9] where both high sensitivity and small assay scale are essential.

Since the lanthanide f−f transitions have a low absorption coefficient because of the Laporte selection rule, sensitized emission is often used to achieve high luminescence.[5] Here, a chromophore incorporated in the ligand (called a sensitizer or an antenna) absorbs excitation light with a large absorption coefficient and transfers its energy to the metal by intersystem crossing, whereby the metal enters the emission state (Figure 1a).[10] By choosing an appropriate antenna, it is possible to obtain a highly emissive lanthanide complex.[10a,11]

Currently, there is growing interest in lanthanide-based luminescence probes for biological applications. Compared with organic fluorophores, however, only a limited number of these probes have so far been reported, though their targets include pH,[12] cations,[13] anions,[14] and others.[15] The lack of a coherent strategy for the design of luminescent sensors slows the development of highly sensitive probes suitable for practical use.

Photoinduced electron transfer (PeT) is a comprehensively reported mechanism for excited-state quenching, in which an electron is transferred from an electron donor moiety to an

(6) (a) Parker, D.; Dickins, R. S.; Puschmann, H.; Crossland, C.; Howard, J. A. K. *Chem. Rev.* **2002**, *102*, 1977−2010. (b) Døssing, A. *Eur. J. Inorg. Chem.* **2005**, 1425−1434.
(7) (a) Hemmilá, I.; Mukkala, V.-M. *Crit. Rev. Clin. Lab. Sci.* **2001**, *38*, 441−519. (b) Lei, W.; Dürkop, A.; Lin, Z.; Wu, M.; Wolfbeis, O. S. *Microchim. Acta* **2003**, *143*, 269−274.
(8) (a) Selvin, P. R. *Annu. Rev. Biophys. Biomol. Struct.* **2002**, *31*, 275−302. (b) Lee, Y. C. *Anal. Biochem.* **2001**, *297*, 123−127. (c) Johansson, M. K.; Cook, R. M.; Xu, J.; Raymond, K. N. *J. Am. Chem. Soc.* **2004**, *126*, 16451−16455.
(9) (a) Yuan, J.; Wang, G.; Majima, K.; Matsumoto, K. *Anal. Chem.* **2001**, *73*, 1869−1876. (b) Mathis, G. *Clin. Chem.* **1993**, *39*, 1953−1959.
(10) (a) Hemmilä, I.; Webb, S. *Drug Discov. Today* **1997**, *9*, 373−381. (b) Karvinen, J.; Hurskainen, P.; Gopalakrishnan, S.; Burns, D.; Warrior, U.; Hemmilä, I. *J. Biomol. Screen.* **2002**, *7*, 223−231. (c) Préaudat, M.; Ouled-Diaf, J.; Alpha-Bazin, B.; Mathis, G.; Mitsugi, T.; Aono, Y.; Takahashi, K.; Takemoto, H. *J. Biomol. Screen.* **2002**, *7*, 267−274.
(11) (a) Alpha, B.; Lehn, J.-M.; Mathis, G. *Angew. Chem., Int. Ed. Engl.* **1987**, *26*, 266−267. (b) Beeby, A.; Faulkner, S.; Parker, D.; Williams, J. A. G. *J. Chem. Soc., Perkin Trans. 2* **2001**, 1268−1273. (11) (a) Li, M.; Selvin, P. R. *J. Am. Chem. Soc.* **1995**, *117*, 8132−8138. (b) Chen, J.; Selvin, P. R. *J. Photochem. Photobiol., A* **2000**, *135*, 27−32. (c) Steemers, F. J.; Verboom, W.; Reinhoudt, D. N.; van der Tol, E. B.; Verhoeven, J. W. *J. Am. Chem. Soc.* **1995**, *117*, 9408−9414. (d) Fu, L.-M.; Wen, X.-F.; Ai, X.-C.; Sun, Y.; Wu, Y.-S.; Zhang, J.-P.; Wang, Y. *Angew. Chem., Int. Ed.* **2005**, *44*, 747−750. (e) Dadabhoy, A.; Faulkner, S.; Sammes, P. G. *J. Chem. Soc., Perkin Trans. 2* **2002**, 348−357. (f) Petoud, S.; Cohen, S. M.; Bünzli, J.-C. G.; Raymond, K. N. *J. Am. Chem. Soc.* **2003**, *125*, 13324−13325. (g) Faulkner, S.; Pope, S. J. A. *J. Am. Chem. Soc.* **2003**, *125*, 10526−10527. (h) Mürner, H.-R.; Chasssat, E.; Thummel, R. P.; Bünzli, J.-C. G. *J. Chem. Soc., Dalton Trans.* **2000**, 2809−2816. (i) Coppo, P.; Duati, M.; Kozhevnikov, V. N.; Hofstraat, J. W.; De Cola, L. *Angew. Chem., Int. Ed.* **2005**, *44*, 1806−1810. (j) Galaup, C.; Carrié, M.-C.; Tisnès, P.; Picard, C. *Eur. J. Org. Chem.* **2001**, 2165−2175. (k) Elbanowski, S. L.; Mąkowska, B.; Hnatejko, Z. *J. Photochem. Photobiol., A* **2002**, *150*, 233−247.
(12) (a) de Silva, A. P.; Gunaratne, H. Q. N.; Rice, T. E. *Angew. Chem., Int. Ed. Engl.* **1996**, *35*, 2116−2118. (b) Parker, D.; Senanayake, P. K.; Williams, J. A. G. *J. Chem. Soc., Perkin Trans. 2* **1998**, 2129−2139. (c) Gunnlaugsson, T.; MacDónaill, D. A.; Parker, D. *J. Am. Chem. Soc.* **2001**, *123*, 12866−12876. (d) Lowe, M. P.; Parker, D.; Reany, O.; Aime, S.; Botta, M.; Castellano, G.; Gianolio, E.; Pagliarin, R. *J. Am. Chem. Soc.* **2001**, *123*, 7601−7609. (e) Gunnlaugsson, T.; Leonard, J. P.; Sénéchal, K.; Harte, A. J. *J. Am. Chem. Soc.* **2003**, *125*, 12062−12063.
(13) (a) de Silva, A. P.; Gunaratne, H. Q. N.; Rice, T. E.; Stewart, S. *Chem. Commun.* **1997**, 1891−1892. (b) Reany, O.; Gunnlaugsson, T.; Parker, D. *J. Chem. Soc., Perkin Trans. 2* **2000**, 1819−1831. (c) Hanaoka, K.; Kikuchi, K.; Kojima, H.; Urano, Y.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 12470−12476.
(14) (a) Dickins, R. S.; Gunnlaugsson, T.; Parker, D.; Peacock, R. D. *Chem. Commun.* **1998**, 1643−1644. (b) Charbonnière, L.; Ziessel, R.; Montalti, M.; Prodi, L.; Zaccheroni, N.; Boehme, C.; Wipff, G. *J. Am. Chem. Soc.* **2002**, *124*, 7779−7788. (c) Atkinson, P.; Bretonniere, Y.; Parker, D. *Chem. Commun.* **2004**, 438−439.
(15) (a) de Sousa, M.; Kluciar, M.; Abad, S.; Miranda, M. A.; de Castro, B.; Pischel, U. *Photochem. Photobiol. Sci.* **2004**, *3*, 639−642. (b) Bobba, G.; Frias, J. C.; Parker, D. *Chem. Commun.* **2002**, 890−891. (c) Best, M. D.; Anslyn, E. V. *Chem.−Eur. J.* **2003**, *9*, 51−57. (d) Lee, K.; Dzubeck, V.; Latshaw, L.; Schneider, J. P. *J. Am. Chem. Soc.* **2004**, *126*, 13616−13617. (e) Hamblin, J.; Abboyi, N.; Lowe, M. P. *Chem. Commun.* **2005**, 657−659.

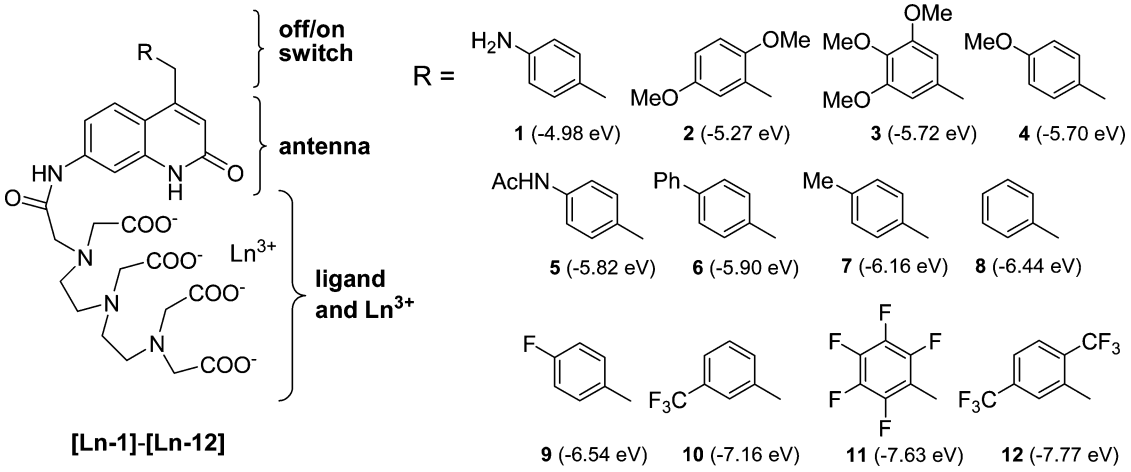

Figure 2. Structures of **[Ln−1]** to **[Ln−12]**. Calculated HOMO energy levels of the off/on switch moiety are also shown.

acceptor moiety.[16] If the electron donor has a high HOMO level, the fluorescence of the electron acceptor is quenched (Figure 1b). The PeT mechanism has been examined in detail as a fluorescence off/on switch for fluorescein derivatives,[17] and it has become possible to rationally develop fluorescein-based fluorescence probes with finely controlled photophysical properties.[18]

In the case of lanthanide complexes, PeT can take place in several ways: ligand to metal,[19] ligand to ligand,[12a,15a,20] extramolecular species to metal or ligand,[21] and others. Among them, intramolecular PeT within the ligand moiety should be most useful in rationally developing a luminescence probe, because using this kind of PeT as a switch eliminates the need to incorporate the reactive moiety in the antenna, which is a great limitation in designing a probe. In other words, it is possible to optimize the reactive moiety for the target species independently of the antenna structure. Surprisingly, this sort of PeT has not been investigated in detail before.

With the aim of establishing a rational and convenient methodology for the development of long-lived luminescence probes, we set out to scrutinize the potent role of PeT within the ligand moiety as a luminescence off/on switch. We have synthesized a series of complexes, **[Ln−X]** (**Ln = Eu**, **Tb**) (**X = 1−12**), the ligands of which consist of three moieties: a chelator, an antenna, and a luminescence on/off switch, of which the latter corresponds to the reactive moiety of the probe. Clear dependence of lanthanide luminescence on the switch HOMO level was observed, which shows that the luminescence intensity of the lanthanide complexes can indeed be controlled by intramolecular PeT within the ligand.

To demonstrate the feasibility of the PeT-based design of luminescence probes, a novel luminescence probe, **[Ln−13]** (**Ln = Tb**, **Eu**), for a protease, microsomal leucine aminopeptidase (LAP), was developed. This complex drastically changes its luminescence property in response to the enzymatic activity of LAP. Applications of the probe for inhibition activity assay, microplate assay, and clinical diagnosis are described. To our knowledge, this is the first functional long-lived luminescence probe for enzymatic activity, controlled by the PeT mechanism.

Results and Discussion

Design and Synthesis of [Ln−X]. To examine the effect of PeT within the ligand moiety, we designed and synthesized a series of complexes **[Ln−X]** (**Ln = Eu**, **Tb**) (**X = 1−12**), which consist of three parts: a Ln^{3+} chelate, an antenna, and a luminescence on/off switch (Figure 2). As a chelator, diethylenetriaminepentaacetic acid (DTPA) was chosen. DTPA forms a highly stable ($K_{ML} = 10^{22.39}$ and $10^{22.71}$ for Eu^{3+} and Tb^{3+}, respectively)[22] complex with a Ln^{3+} ion, with a coordination number of 8.[23]

It also prevents solvent water molecules from coupling away energy from the excited state of the lanthanide ion. As an antenna, 7-amino-4-methyl-2(1*H*)-quinolinone (cs124) was selected, because the DTPA-amide of this compound works as an efficient sensitizer for both Tb^{3+} and Eu^{3+} in aerated water.[11a,24] The luminescence on/off switch is a substituted benzene of appropriate electron density, which was linked to the methyl group of the antenna to avoid conjugative coupling of the two aromatic rings. We have synthesized 12 compounds as described in detail in the Supporting Information, followed by complexation with Ln^{3+}.

Calculation of HOMO Energy Level. The likelihood of PeT can be judged from the change in free energy (ΔG_{eT}), which is calculated from the Rehm−Weller eq 1

$$\Delta G_{eT} = E_{ox} - E_{red} - \Delta E_{0,0} - w_p \qquad (1)$$

where E_{ox} and E_{red} are the oxidation and reduction potentials of the electron donor (on/off switch moiety) and acceptor

(16) Callan, J. F.; de Silva, A. P.; Magri, D. C. *Tetrahedron* **2005**, *61*, 8551−8588.
(17) (a) Ueno, T.; Urano, Y.; Setukinai, K.; Takakusa, H.; Kojima, H.; Kikuchi, K.; Ohkubo, K.; Fukuzumi, S.; Nagano, T. *J. Am. Chem. Soc.* **2004**, *126*, 14079−14085. (b) Miura, T.; Urano, Y.; Tanaka, K.; Nagano, T.; Ohkubo, K.; Fukuzumi, S. *J. Am. Chem. Soc.* **2003**, *125*, 8666−8671.
(18) (a) Kenmoku, S.; Urano, Y.; Kanda, K.; Kojima, H.; Kikuchi, K.; Nagano, T. *Tetrahedron* **2004**, *60*, 11067−11073. (b) Urano, Y.; Kamiya, M.; Kanda, K.; Ueno, T.; Hirose, K.; Nagano, T. *J. Am. Chem. Soc.* **2005**, *127*, 4888−4894. (c) Kamiya, M.; Urano, Y.; Ebata, N.; Yamamoto, M.; Kosuge, J.; Nagano, T. *Angew. Chem., Int. Ed.* **2005**, *44*, 5439−5441.
(19) (a) Abu-Saleh, A.; Meares, C. F. *Photochem. Photobiol.* **1984**, *39*, 763−769. (b) Aime, S. et al. *J. Chem. Soc., Dalton Trans.* **1997**, 3623−3636. (c) Dickins, R. S.; Howard, J. A. K.; Maupin, C. L.; Moloney, J. M.; Parker, D.; Riehl, J. P.; Siligardi, G.; Williams, J. A. G. *Chem.—Eur. J.* **1999**, *5*, 1095−1105.
(20) Parker, D. *Coord. Chem. Rev.* **2000**, *205*, 109−130.
(21) Poole, R. A.; Bobba, G.; Cann, M. J.; Frias, J.-C.; Parker, D.; Peacock, R. D. *Org. Biomol. Chem.* **2005**, *3*, 1013−1024.
(22) Moeller, T.; Thompson, L. C. *J. Inorg. Nucl. Chem.* **1962**, *24*, 499−510.
(23) Caravan, P.; Ellison, J. J.; McMurry, T. J.; Lauffer, R. B. *Chem. Rev.* **1999**, *99*, 2293−2352.
(24) Selvin, P. R.; Jancarik, J.; Li, M.; Hung, L.-W. *Inorg. Chem.* **1996**, *35*, 700−705.

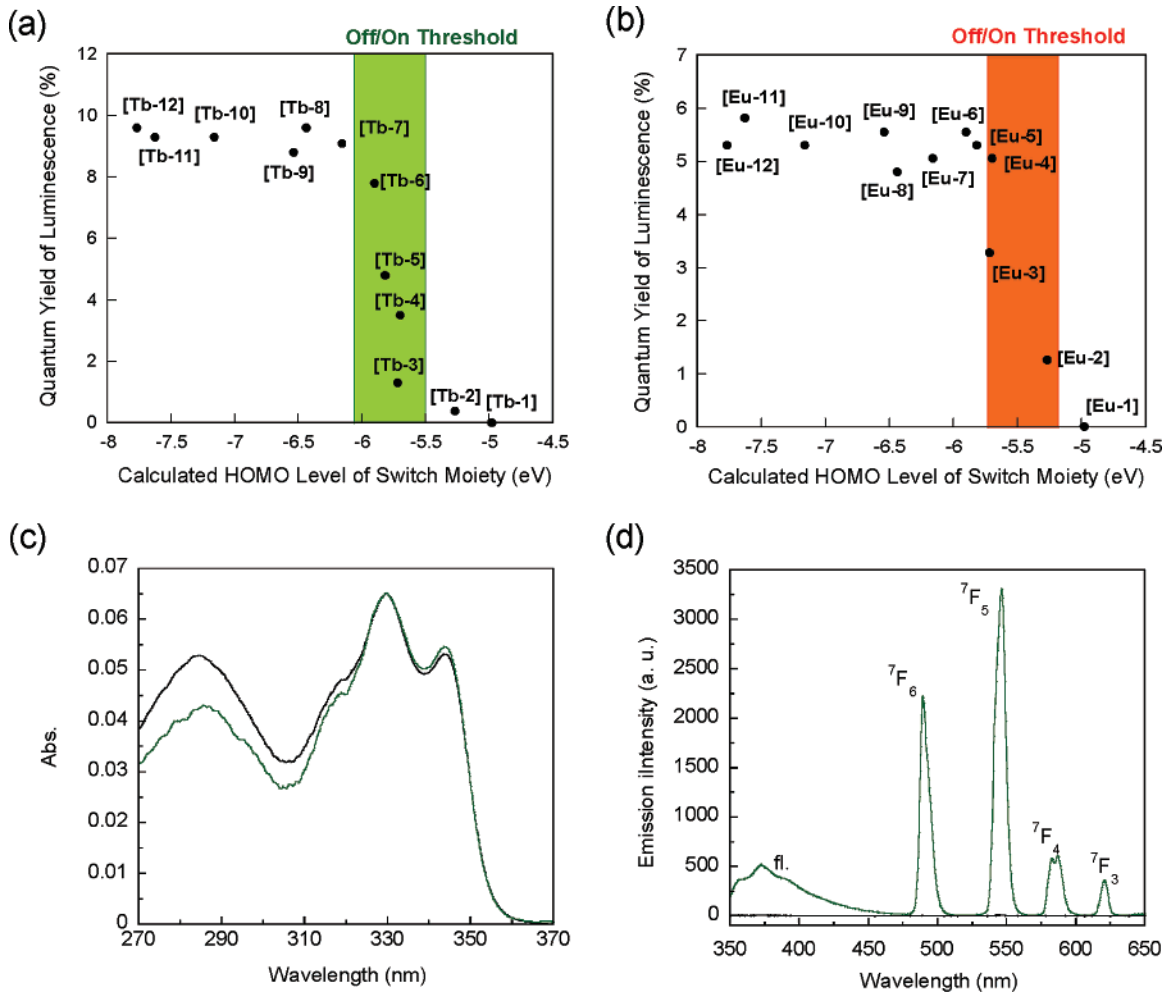

Figure 3. Spectroscopic properties of **[Ln−X]**. Plot of luminescence intensity as a function of the calculated HOMO level of the switch moiety in the cases of (a) Tb^{3+} and (b) Eu^{3+} complexes. (c) Absorption and (d) emission spectra of **[Tb−8]** (green line) and **[Tb−1]** (black line) 5 μM in aqueous solution (100 mM HEPES buffer, pH 7.3, $I = 0.1$ ($NaNO_3$)). Excitation wavelength was 330 nm. The attribution of each band is shown in the figure (see text).

(antenna moiety), $\Delta E_{0,0}$ is the singlet excited energy, and w_p is the work term for the charge separation state.[25] If a fixed acceptor is used, low E_{ox} leads to a negative Δ_{eT} value and facilitates the electron transfer, as can easily be understood from eq 1. Hence, the fluorescence intensity of the compound primarily depends on the oxidation potential of the electron donor moiety. This parameter can be readily evaluated from the HOMO energy level of the donor moiety, as shown for fluorescein derivatives.[18a,b]

Our working hypothesis was that the strategy used in fluorescein derivatives would also be applicable to the synthesized lanthanide complexes **[Ln−X]**. We anticipated that, provided that the HOMO level of the switch moiety is high enough to induce PeT to the antenna, the singlet excited state of the antenna would be quenched, resulting in no luminescence from Ln^{3+} (Figure 1c). In contrast, a complex with a switch having a low HOMO level would not suffer from this quenching mechanism and would emit strong luminescence (Figure 1d).

Bearing this in mind, the HOMO energy level of the luminescence on/off switch of **1**−**12** was calculated by the DFT method using the B3LYP/6-31G(d) basis set. The results are shown in Figure 2. The values cover a wide range from −4.98 eV of **1** to −7.77 eV of **12**.

(25) Rehm, D.; Weller, A. *Isr. J. Chem.* **1970**, *8*, 259−271.

Photochemical Properties of [Ln−X]. For each synthesized complex, the quantum yield of luminescence in neutral aqueous buffer was determined and plotted against the calculated HOMO energy level of the switch moiety (Figure 3). As expected, a clear dependence of quantum yield on the HOMO level was observed for both Tb^{3+} and Eu^{3+} complexes. The threshold level of luminescence on and off switching was shown to be around −5.8 eV for Tb and −5.5 eV for Eu. Only the complexes with a HOMO level of the switch moiety below the threshold have strong luminescence, and their quantum yields are similar to those of the complexes without the switch moiety (12.1% and 4.3% for Tb^{3+} and Eu^{3+} complexes, respectively). The slight difference of the threshold level between Tb^{3+} and Eu^{3+} is probably due to the effect of the metal on the π-system of the antenna moiety. Such a phenomenon has been suggested before in a complex in which the antenna directly binds to the lanthanide ion.[12a] Considering that the 7-amide group of cs124-DTPA-Ln is known to participate in metal binding,[24] our result is not surprising.

To scrutinize the mechanism of quenching, the luminescence and chemical properties of **[Ln−X]** were investigated. For clarity, the absorption and emission spectra of **[Tb−8]** (PeT off state) and **[Tb−1]** (PeT on state) are shown in Figure 3c and 3d, respectively. The UV−vis absorption spectra (over

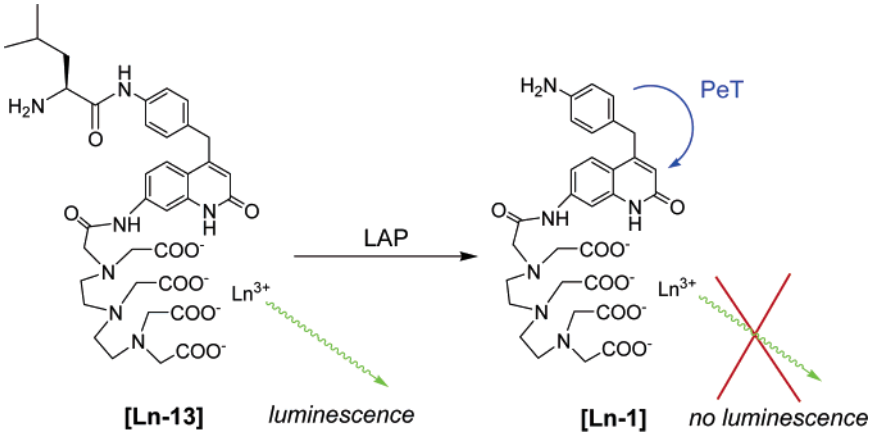

Figure 4. Schematic representation of the probe. **Ln** = **Tb**, **Eu**.

310 nm) did not change among the complexes, showing that the ground state of the antenna is not affected by the switch moiety. For **[Tb-8]**, short-lived emission of the antenna moiety (labeled as fl. in Figure 3d) was detected at around 370 nm, as well as four luminescence bands of Tb^{3+} at 490, 546, 583, and 621 nm. These bands correspond to the transition from the $^5D_4$ excited state to the $^7F_6$, $^7F_5$, $^7F_4$, and $^7F_3$ ground state, respectively. No emission, however, was observed from **[Tb−1]**, including the fluorescence of the antenna at around 370 nm. In the case of Eu^{3+} complexes, essentially identical results were obtained, except that the observed transition from the Eu^{3+} excited state occurred from $^5D_0$ to ^7F_J (J = 0, 1, 2, 3, 4) (Figure S1). This quenching of antenna fluorescence, in cooperation with the luminescence from Ln^{3+}, indicates a presence of a quenching pathway, such as PeT, to the singlet excited state of the antenna moiety.

Other parameters listed in Table S1 (Tb^{3+} complexes) and S2 (Eu^{3+} complexes) are also consistent with the idea that the luminescence of **[Ln−X]** is controlled by PeT from the off/on switch moiety to the antenna singlet state. The triplet energy level of the antenna, calculated from the phosphorescence spectra measured in MeOH/EtOH = 1:1 at 77 K did not change at all, which excludes the possibility that the quenching of luminescence is due to a change of the nature of the antenna triplet state. In addition, either in H_2O or in D_2O, the luminescence lifetimes of the complexes with different switches did not vary, demonstrating that the excited state of the lanthanide ion remains intact. It is well-known that the O−H oscillator of solvent coordinated to the metal center can quench the luminescence from Ln^{3+}.[26,27] For every complex, however, the number of coordinated water molecules (q values) at the metal center was estimated to be approximately 1, using the equation proposed by Parker et al.[27] This shows that the off/on switch does not change this nonradiative decay of Ln^{3+} by bound water molecules.

Figure 3a and 3b provide us with highly significant information for designing a luminescence probe. To develop a practical probe with a large change of luminescence in response to the target, what we need to do is to find a reactive moiety whose HOMO level is changed across the threshold by the reaction. In other words, we can screen and optimize the structure of the probe *in silico*. Compared with the previously exploited *trial and error* approach, this rational method should have many advantages, including savings of cost and time.

Design and Photochemical Properties of an LAP Probe. Based on the above findings, we set out to develop a protease probe based on PeT. A protease is an enzyme which cleaves a specific peptide bond of a substrate peptide or protein. There are enormous numbers of enzymes in this category,[28] many of which are related to various diseases.[29] Although several protease substrates for chromogenic and fluorogenic assays have been reported,[30] none of them has a long-lived luminescence except for a few[31] based on RET (resonance energy transfer). In contrast to RET-based probes, which require two chromophores at appropriate sites, probes using PeT should provide simple and robust assays and should also be applicable to exopeptidases.

Among the proteases, microsomal leucine aminopeptidase (LAP) was selected as the target enzyme. LAP (EC 3.4.11.2, also known as aminopeptidase M or aminopeptidase N) is a widespread exopeptidase, which removes N-terminal amino acids (preferably leucine and alanine) from almost all unsubstituted oligopeptides.[28] It is well-known that LAP plays important roles in tumor-cell invasion,[32] tumor metastasis,[33] and maturation of MHC class I epitopes.[34] Although chromogenic and fluorogenic substrates, L-pNA[35] and L-MCA,[36]

(26) Horrocks, W. DeW., Jr.; Sudnick, D. R. *Acc. Chem. Res.* **1981**, *14*, 384−392.
(27) Beeby, A.; Clarkson, I. M.; Dickins, R. S.; Faulkner, S.; Parker, D.; Royle, L.; de Sousa, A. S.; Williams, J. A. G.; Woods, M. *J. Chem. Soc., Perkin Trans. 2* **1999**, 493−503.
(28) Barrett, A. J.; Rawlings, N. D.; Woessner, J. F. *Handbook of Proteolytic Enzymes*, 2nd ed.; Elsevier Academic Press: Amsterdam, The Netherlands, 2004.
(29) (a) Southan, C. *Drug Discov. Today* **2001**, *6*, 681−688. (b) Artal-Sanz, M.; Tavernarakis, N. *FEBS Lett.* **2005**, *579*, 3287−3296. (c) Singh, R. B.; Dandekar, S. P.; Elimban, V.; Gupta, S. K.; Dhalla, N. S. *Mol. Cell. Biochem.* **2004**, *263*, 241−256.
(30) (a) Erlanger, B. F.; Kokowsky, N.; Cohen, W. *Arch. Biochem. Biophys.* **1961**, *95*, 271−278. (b) Zimmerman, M.; Yurewicz, E.; Patel, G. *Anal. Biochem.* **1976**, *70*, 258−262. (c) Leytus, S. P.; Patterson, W. L.; Mangel, W. F. *Biochem. J.* **1983**, *215*, 253−260.
(31) (a) Karvinen, J.; Elomaa, A.; Mäkinen, M.-L.; Hakala, H.; Mukkala, V.-M.; Peuralahti, J.; Hurskainen, P.; Hovinen, J.; Hemmilä, I. *Anal. Biochem.* **2004**, *325*, 317−325.(b) Karvinen, J.; Laitala, V.; Mäkinen, M.-L.; Mulari, O.; Tamminen, J.; Hermonen, J.; Hurskainen, P.; Hemmilä, I. *Anal. Chem.* **2004**, *76*, 1429−1436.
(32) Saiki, I.; Fujii, H.; Yoneda, J.; Abe, F.; Nakajima, M.; Tsuruo, T.; Azuma, I. *Int. J. Cancer* **1993**, *54*, 137−143.
(33) Fujii, H.; Nakajima, M.; Saiki, I.; Yoneda, J.; Azuma, I.; Tsuruo, T. *Clin. Exp. Metastasis* **1995**, *13*, 337−344.
(34) Saric, T.; Chang, S.-C.; Hattori, A.; York, I. A.; Markant, S.; Rock, K. L.; Tsujimoto, M.; Goldberg, A. L. *Nat. Immunol.* **2002**, *3*, 1169−1176.
(35) Tuppy, H.; Wiesbauer, U.; Wintersberger, E. *Hoppe-Seyler's Z. Physiol. Chem.* **1962**, *329*, 278−288.
(36) Kanaoka, Y.; Takahashi, T.; Nakayama, H. *Chem. Pharm. Bull.* **1977**, *25*, 362−363.
(37) Melhuish, W. H. *J. Phys. Chem.* **1961**, *65*, 229−235.

Table 1. Photochemical Properties of the Probes

	abs_{max} (nm)	ϵ	em_{max} (nm)	Φ_{lum}[a] (%)	τ_{H_2O} (ms)	τ_{D_2O} (ms)	q[b]
[Tb−13]	330	13 000	547	6.8	1.12	1.70	1.20
[Tb−1]	330	13 000	547	0.01	n.d.[c]	n.d.[c]	n.d.[c]
[Eu−13]	330	13 000	614	4.8	0.62	2.10	0.96
[Eu−1]	330	13 000	614	0.01	n.d.[c]	n.d.[c]	n.d.[c]

[a] Quantum yields were determined using quinine sulfate ($\Phi = 0.546$ in 1 N H_2SO_4)[37] for Tb and $[Ru(bpy)_3]Cl_2$ ($\Phi = 0.028$ in air-equilibrated water)[38] for Eu complexes as standards. Measurements were performed in 100 mM HEPES buffer (pH 7.4) at 25 °C. [b] q values were estimated using the equation $q = 5(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - 0.06)$ (for Tb) and $q = 1.2(\tau_{H_2O}^{-1} - \tau_{D_2O}^{-1} - 0.25 - 0.075)$ (for Eu).[27] [c] Not determined.

respectively, have been reported, there is so far no substrate suitable for TRF measurement.

The design of the probe is shown in Figure 4. The substrate peptide sequence of LAP, L-Leu, was attached through a peptide bond to the amino group of the luminescence off/on switch moiety of **[Ln−1]** (for detailed synthetic procedure, see Supporting Information). The synthesized compound, **[Ln−13]**, was expected to have strong luminescence, because the HOMO energy level of its switch moiety (−5.89 eV) was similar to that of **[Ln−6]**. After the cleavage of the peptide by LAP, **[Ln−13]** would be converted to **[Ln−1]**, whose luminescence would be quenched by PeT.

The photochemical properties of **[Ln−13]**, compared with those of **[Ln−1]**, were summarized in Table 1. As expected, the parameters do not differ from each other except for the quantum yields of luminescence. The quantum yields of **[Ln−13]** were close to those of **[Ln−6]**, indicating the accuracy of the prediction from the calculated HOMO energy level. For both Tb^{3+} and Eu^{3+}, the luminescence intensity of the complexes before and after the LAP-mediated reaction differed by approximately 500-fold, indicating that this probe is potentially useful for the sensitive detection of LAP activity. Because the quantum yield of **[Tb−13]** is higher than that of the corresponding Eu^{3+} complex, most of the following experiments were performed with **[Tb−13]**.

Enzymatic Reaction and Assessment of Inhibitory Activity. When the probe was reacted with LAP, the emission from Ln^{3+}, as well as that from the antenna, decreased in a time-dependent manner (Figure 5) until complete quenching was achieved (Figure S2). This decrease was not observed if the enzyme was absent or if the **[Ln−13]** complex was replaced with **[Ln−5]** (Figure S3). In addition, other proteases, such as trypsin, chymotrypsin, elastase, caspase-3, and cathepsin B, did not affect the luminescence of the probe (for trypsin, data are shown in Figure S3), which shows that **[Ln−13]** is a specific probe for LAP activity. The conversion of **[Tb−13]** to **[Tb−1]** was confirmed by analytical HPLC (Figure S4).

Kinetic parameters of **[Tb−13]** were determined and compared with those of commercially available substrates, L-pNA and L-MCA (Table S3). The K_m and k_{cat} of **[Tb−13]** were found to be 4.18×10^5 M and 29.3 s^{-1}, respectively. The k_{cat}/K_m value was 7.01×10^5 (M^{-1} s^{-1}), virtually identical with those of L-pNA and L-MCA (7.17×10^5 and 6.13×10^5 M^{-1} s^{-1}). This result indicates that the reaction rates are almost the same, when the probe concentration is low.

One possible application of a spectroscopic probe for a protease is to evaluate inhibitory activities of candidate compounds. Indeed, protease inhibitors are widely used as therapeutic drugs.[39] In particular, LAP inhibitors are suggested to have antitumor angiogenesis activity.[39b] Thus, we have investigated the applicability of **[Tb−13]** for the assessment of inhibitory activity. Several compounds are known to inhibit the activity of LAP, including actinonin,[40] amastatin,[41] and bestatin.[42] The inhibitory activities of these three inhibitors were probed with L-pNA, L-MCA, and **[Tb−13]**, respectively, and the inhibition constants K_i were determined on the assumption that these inhibitors were noncompetitive (Table S4).[43] Similar results were obtained regardless of the probes used.

TRF Assay on Microplate. Microplates (96-well, 384-well, etc.) are an indispensable tool for biological and environmental assays. They can handle a large number of samples simultaneously with a small sample volume (microliter order) and are suitable for automated assay systems. They are, however, more likely to suffer from autofluorescence of the device and scattered light. Therefore, TRF measurements with low background signals are expected to be highly advantageous compared with the use of organic fluorescent compounds.[9a,44]

After confirming that LAP activity on a 96-well plate could be monitored with 2.5 μM **[Tb−13]** by TRF measurement (Figure S5), we conducted experiments with lower concentrations of **[Tb−13]**, such as at nanomolar level. The experiments were repeated using L-MCA to compare the results. At 50 nM, **[Tb−13]** could clearly detect the presence of inhibitor ($P < 0.001$ by Student's t-test), while L-MCA failed to show a statistically significant difference of fluorescence intensity ($P > 0.05$) between with and without inhibitor, due to the large background signal (Figure 6).

We also performed experiments with smaller amounts of LAP. With **[Tb-13]**, as little as 1 μU of LAP could be detected (Figure S6a) in a concentration-dependent manner. With L-MCA, however, no increase of fluorescence intensity was observed when the amount of LAP was lower than 50 μU, presumably due to the influence of the background signal (Figure S6b).

Although some influence of the apparatus cannot be excluded, these results suggest that TRF measurements with **[Tb-13]** offer higher reproducibility and sensitivity than conventional fluorescence assays in a system with a high background signal level, such as microplates.

Application for Clinical Diagnosis. LAP is a clinically significant enzyme, and elevated levels of LAP activity in serum are associated with diseases of the liver, bile duct, and pancreas.[45] As LAP activity in serum renders valuable information for clinical diagnosis, it is often quantified in hospitals. To

(38) Nakamaru, K. *Bull. Chem. Soc. Jpn.* **1982**, *55*, 2697−2705.

(39) (a) Leung, D.; Abbenante, G.; Fairlie, D. P. *J. Med. Chem.* **2000**, *43*, 305−341.(b) Aozuka, Y.; Koizumi, K.; Saitoh, Y.; Ueda, Y.; Sakurai, H.; Saiki, I. *Cancer Lett.* **2004**, *216*, 35−42.

(40) Umezawa, H.; Aoyagi, T.; Tnanaka, T.; Suda, H.; Okuyama, A.; Naganawa, H.; Hamada, M.; Takeuchi, T. *J. Antibiotics* **1985**, *38*, 1629−1630.

(41) Aoyagi, T.; Tobe, H.; Kojima, F.; Hamada, M.; Takeuchi, T.; Umezawa, H. *J. Antibiotics* **1978**, *31*, 636−638.

(42) Umezawa, H.; Aoyagi, T.; Suda, H.; Hamada, M.; Takeuchi, T. *J. Antibiotics* **1976**, *29*, 97−99.

(43) Barclay, R. K.; Phillips, M. A. *Biochem. Biophys. Res. Commun.* **1980**, *96*, 1732−1738.

(44) Gudgin Dickson, E. F.; Pollak, A.; Diamandis, E. P. *J. Photochem. Photobiol., B* **1995**, *27*, 3−19.

(45) (a) Szasz, G. *Am. J. Clin. Path.* **1967**, *47*, 607−613. (b) Shay, H.; Sun, D. C. H.; Siplet, H. *Am. J. Digest. Dis.* **1960**, *5*, 217−232. (c) Pineda, E. P.; Goldbarg, J. A.; Banks, B. M.; Rutenburg, A. M. *Gastroenterology* **1960**, *38*, 698−712.

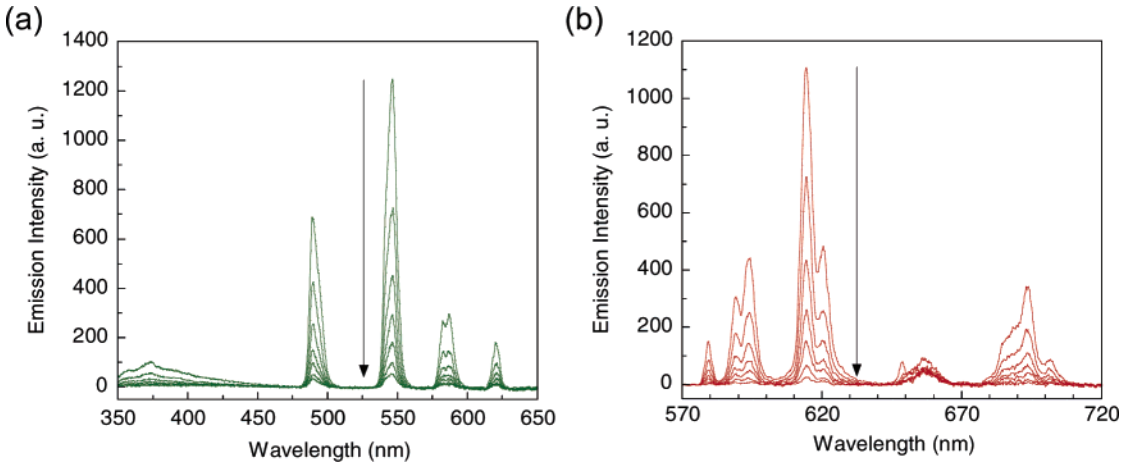

Figure 5. Reaction of **[Ln−13]** with LAP. Emission spectra of 2 μM (a) **[Tb−13]** and (b) **[Eu−13]** at 0, 5, 10, 15, 20, 30, and 60 min after the addition of LAP (0.01 U). The reaction was performed in 100 mM Tris-HCl buffer (pH 7.4) at 37 °C. The excitation wavelength was 330 nm.

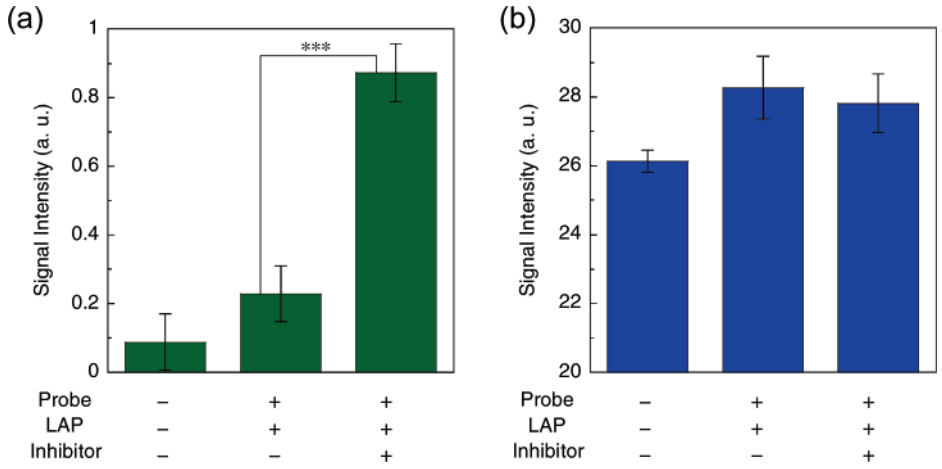

Figure 6. Microplate assay of LAP (0.002 U) with 50 nM (a) **[Tb−13]** (TRF measurement) and (b) L-MCA. Amastatin (10 μM) was added as an inhibitor. Fluorescence intensity (ex./em. = 325/545 nm for (a) and 380/440 nm for (b)) was recorded 60 min after the initiation of the reaction. Data are shown as mean ± SEM (n = 5). *** indicates $P < 0.001$.

examine the feasibility of applying **[Tb-13]** for clinical diagnosis, we conducted experiments using human sera. This is a challenging experimental condition, since serum contains hundreds of biological compounds and ions. It absorbs more than 90% of excitation light (at 330 nm), emitting a strong background fluorescence. Using TRF measurements, LAP activities in sera were quantified in both normal volunteers and patients with cancer (5 persons each). As can be seen in Figure 7, LAP activity was 1.9-fold higher in sera from patients than in sera from normal persons ($P < 0.01$ by Student's t-test). The results obtained with this method were shown to have a high linear correlation (r = 0.94) with commercially performed measurements of the same samples, conducted with a chromogenic substrate (Figure S7). Moreover, the coefficient of variation (CV) for normal patients was 17.7, about half that of the chromogenic method (30.0). The luminescence intensity of **[Tb−13]** did not change at all when amastatin, a known inhibitor of LAP, was added to the serum, which confirmed that **[Tb−13]** specifically measured the LAP activity of human serum (Figure S8).

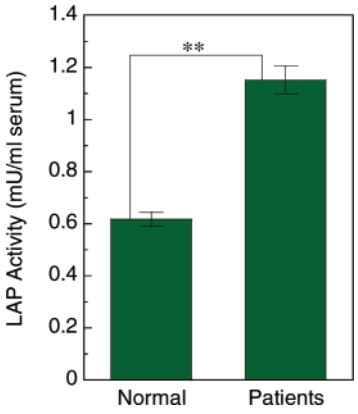

Figure 7. Difference of LAP activity in human sera between normal subjects and patients with cancer. Assay was performed in 10% serum with 1 μM **[Tb−13]**. Luminescence intensity (ex./em. = 330/545 nm) was measured for the initial 10 min using TRF measurement and converted to enzyme activity. In this case, 1 U was defined as the amount of protein which is required to hydrolyze 1 μmol of **[Tb−13]** per min at 37 °C, pH 7.4. Data are shown as mean ± SEM (n = 5). ** indicates a significant difference, $P < 0.01$.

Compared with colorimetric assays, a fluorescence assay has many advantages. For instance, whereas fluorescence assays can be performed at submicromolar concentrations of the probes, colorimetric assays require millimolar levels. Another advantage is that simultaneous detection of different targets is possible using multiple fluorescent probes with different excitation or emission wavelengths. As one of the major disadvantages, i.e., background fluorescence, can be eliminated by means of TRF

measurements (see previous section), it is expected that TRF assays using lanthanide complexes such as **[Tb−13]** will be of great practical value.

Conclusions

We have designed and synthesized a series of lanthanide complexes, each consisting of three moieties: a lanthanide chelate, a luminescence off/on switch, and an antenna. As we had anticipated, quenching of lanthanide luminescence was observed as the HOMO energy level of the switch moiety became higher. By careful examination of the photochemical properties of these complexes, we demonstrated that the quenching of the complexes observed here is due to intramolecular PeT from the switch moiety to the antenna. This is the first report in which the effectiveness of intramolecular PeT as a mechanism for luminescence off/on switch has been studied. The luminescence off/on threshold for both Tb^{3+} and Eu^{3+} complexes was determined for the first time; this is critical information for the design of luminescence probes. These findings are an important step in establishing a rational method for the development of luminescence-based chemosensors.

By applying this novel approach of calculating the HOMO level of the switch moiety, we developed a luminescence probe for LAP, **[Ln−13]**. It was confirmed to be a sensitive probe for monitoring LAP activity on microplates, where the high background fluorescence could be eliminated by TRF measurement. **[Ln−13]** was also shown to be potentially useful in clinical diagnosis. It is noteworthy that the peptide sequence of **[Tb−13]** can be changed as desired, so that one can easily develop a luminescence probe for any desired protease.

The greatest advantage of our PeT-based approach is that the luminescence intensity of the complex can be predicted to a large extent by calculating the HOMO energy level of the switch moiety. The protease probe described here is not the only application of this approach. By selecting appropriate switch moieties, it can in principle be applied to every target species, including metal ions, enzymes, ROS, and so on.

Recently, a few groups have reported time-resolved luminescence imaging of lanthanide complexes.[46] For instance, we have developed a luminescence microscopy system which can obtain time-resolved images of cells, and we successfully visualized intracellular Zn^{2+} with virtually no background.[47] The development of rationally designed probes for molecules of interest, in combination with these imaging systems, will open a new frontier in fluorescence imaging.

Experimental Section

Materials. Boc-L-Leu-OH, HBTU, and HOBT·H_2O were obtained from Watanabe Chemical Industries, Ltd. (Japan). All other reagents for organic synthesis were purchased from Tokyo Kasei Co., Ltd. (Tokyo, Japan) or Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Microsomal LAP (= aminopeptidase M) was purchased from Pierce Biochemistry, Inc. (Rockford, IL). Unless otherwise stated, 1 U is defined as the amount of protein which is required to hydrolyze 1 μmol of L-pNA per min at 25 °C, pH 7.2. Trypsin (from bovine pancreas), actinonin, amastatin hydrochloride, and bestatin hydrochloride were purchased from SIGMA (St. Louis, MO). Leu-pNA was purchased from Peptide Institute, Inc. (Osaka, Japan). Leu-MCA was purchased from Bachem AG (Bubendorf, Switzerland). Silica gel column chromatography was performed using silica gel 60N (Kanto Kagaku Co., Ltd., (Tokyo, Japan)). ODS column chromatography was performed using Chromatorex-ODS (Fuji Silysia Chemical Ltd., Japan).

Instruments. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL JNM-LA300. Mass spectra were recorded on a JEOL JMS-SX102A mass spectrometer (EI^+), a JEOL JMS-700 mass spectrometer (FAB^+), or a JEOL JMS-T100LC (ESI^+, ESI^-). Preparative HPLC purification was performed on a reversed-phase ODS column (GL Sciences (Tokyo, Japan), Inertsil Prep-ODS 30 mm × 250 mm) fitted on a JASCO PU-1587 HPLC system. UV−visible spectra were obtained on a Shimadzu UV-1600 (Tokyo, Japan). Fluorescence and phosphorescence spectroscopic studies were performed with a Hitachi F4500 (Tokyo, Japan). Time-resolved luminescence spectra and lanthanide luminescence lifetimes were obtained using a Perkin-Elmer LS-55 (Beaconsfield, Buckinghamshire, England). Luminescence assays on 96-well plates were performed using a plate reader instrument coupled with a Perkin-Elmer LS-55.

UV−visible Absorption Spectral Measurements. Absorption spectra of the complexes were measured at 5 μM in 100 mM HEPES buffer (pH 7.3, $I = 0.1$ ($NaNO_3$)). Each sample contained less than 0.1% DMSO as a cosolvent. Measurements were performed in 1 cm quartz cells at 25 °C.

Luminescence Spectral Measurements. Luminescence spectra of the complexes without a delay time were measured at 5 μM in 100 mM HEPES buffer (pH 7.3, $I = 0.1$ ($NaNO_3$)), following excitation at 330 nm. Each sample contained less than 0.1% DMSO as a cosolvent. Measurements were performed in 1 cm quartz cells at 25 °C. The excitation slit width was 2.5 nm (for Tb^{3+} complexes) or 5 nm (for Eu^{3+} complexes). The emission slit width was 2.5 nm. The photomultiplier voltage was 950 V.

Time-Delayed Luminescence Spectral Measurements. Time-delayed luminescence spectra of **[Ln−13]** (**Ln = Tb**, **Eu**) (5 μM) were measured in 100 mM HEPES buffer (pH 7.3, $I = 0.1$ ($NaNO_3$)), with a delay time of 50 μs and a gate time of 2.0 ms, after a 10 μs pulsed excitation at 330 nm. Each sample contained less than 0.1% DMSO as a cosolvent. Measurements were performed in 1 cm quartz cells at 25 °C. The excitation slit width was 2.5 nm (for Tb^{3+} complexes) or 5 nm (for Eu^{3+} complexes). The emission slit width was 2.5 nm.

Quantum Yield Measurements. Quantum yields of the complexes were estimated by a relative method with reference to a luminescence standard. For Tb^{3+} complexes, quinine sulfate ($\Phi = 0.546$ in 1 N H_2SO_4)[37] was used as a standard. For Eu^{3+}, $[Ru(bpy)_3]Cl_2$ ($\Phi = 0.028$ in air-equilibrated water)[38] was used. The quantum yields were calculated by eq 2,

$$\Phi_x/\Phi_{st} = [A_{st}/A_x][n_x^2/n_{st}^2][D_x/D_{st}] \quad (2)$$

where Φ is the quantum yield (subscript "st" stands for the reference, and "x", for the sample), A is the absorbance at the excitation wavelength, n is the refractive index (if both spectra are obtained in the aqueous solution, this term is canceled), and D is the area under the luminescence spectra on an energy scale. The sample and the reference were excited at the same wavelength (330 nm), where the absorbance was kept lower than 0.07. The luminescence spectra were measured at 25 °C in 100 mM HEPES buffer (pH 7.3, $I = 0.1$ ($NaNO_3$)) containing less than 0.1% DMSO as a cosolvent. All spectra were obtained with a Hitachi F4500 spectrofluorometer.

Estimation of the Lowest Triplet Energy Level. Lowest triplet energy levels (T_1) were calculated from the wavelength of the first peak of phosphorescence spectra. Phosphorescence spectra of **[Gd−X]** (**X = 1−12**) (20 μM) were measured at 77 K in MeOH/EtOH = 1:1, following excitation at 330 nm. Experiments were performed with

(46) (a) Faulkner, S.; Pope, S. J. A.; Burton-Pye, B. P. *Appl. Spectrosc. Rev.* **2005**, *40*, 1−31. (b) Weibel, N.; Charbonnière, L. J.; Guardigli, M.; Roda, A.; Ziessel, R. *J. Am. Chem. Soc.* **2004**, *126*, 4888−4896. (c) Charbonnière, L.; Ziessel, R.; Guardigli, M.; Roda, A.; Sabbatini, N.; Cesario, M. *J. Am. Chem. Soc.* **2001**, *123*, 2436−2437.

(47) Hanaoka, K.; Kikuchi, K.; Kobayashi, S.; Nagano, T. Manuscript in preparation.

a Hitachi F4500 equipped with a low-temperature phosphorescence measurement unit.

Luminescence Lifetime Measurements. Luminescence lifetimes were measured in both H_2O (100 mM HEPES buffer, pH 7.4) and D_2O (100 mM HEPES buffer, pD 7.4). The luminescence intensity (ex. 330 nm, em. 545 nm (Tb^{3+} complexes) or 615 nm (Eu^{3+} complexes)) at every 10 μs after pulsed excitation was measured using a 10 μs gate time. Data were fitted to a single-exponential decay curve (eq 3), where I and I_0 are the luminescence intensity at time t and time 0, respectively, and τ is the luminescence lifetime.

$$I = I_0 \exp(-t/\tau) \tag{3}$$

Enzymatic Reaction with LAP. All reactions were performed in a quartz cell at 37 °C. LAP (0.01 U) was dissolved in 100 mM Tris-HCl buffer (pH 7.4) and incubated for 5 min, before 2 μM **[Ln−13]** (**Ln = Tb**, **Eu**) was added. The reaction volume was 3.0 mL, and the solution contained less than 0.1% DMSO as a cosolvent. Luminescence spectra were measured at every 5 min after the addition of the probe without a delay time. Control experiments were performed by replacing LAP or **[Ln−13]** with other reagents.

Estimation of Inhibitory Activity. Various amounts of inhibitors were added to 100 mM Tris-HCl buffer (pH 7.4) along with LAP (0.01 U), and the mixtures were incubated for 5 min at 37 °C, followed by the addition of **[Tb−13]** (2 μM). The total reaction volume was 3.0 mL, and the solution contained less than 0.1% DMSO as a cosolvent. Luminescence intensity (ex. 330 nm, em. 545 nm) was measured continuously up to 5 min at 37 °C. The initial velocity of the reaction was calculated from the change of intensity during the first 3 min, where the luminescence decreased in a linear fashion. Inhibitory activity (%) was determined by comparing the velocity in the presence and absence of the inhibitor. Studies using Leu-MCA were performed with the same procedure except for the measured wavelength (ex. 380 nm, em. 440 nm). For Leu-pNA, probe concentration was 20 μM, and the absorbance at 405 nm was recorded. The inhibition constant (K_i) was calculated from the following equation (eq 4), where v = reaction velocity in the presence of inhibitor, v_0 = reaction velocity in the absence of inhibitor, and [I] = inhibitor concentration.

$$\frac{v}{v_0} = \frac{1}{1 + [\mathrm{I}]/K_i} \tag{4}$$

Determination of Kinetic Parameters. Various concentrations of the probes (= Leu-pNA, Leu-MCA, **[Tb−13]**) were dissolved in 100 mM Tris-HCl buffer (pH 7.4). LAP (0.001 U/ml, final concentration) was added to the solution, and the luminescence intensity (or absorbance) was recorded continuously as described above. The initial reaction velocity was calculated, plotted against probe concentration, and fitted to a Michaelis−Menten curve. K_m and k_{cat} were determined by the least-squares method.

Analytical HPLC. To confirm that enzymatic cleavage of the leucine moiety had taken place, analytical RP-HPLC was performed using an Inertsil 3 ODS column (4.6 mm × 250 mm, GL Sciences, Japan), fitted on a JASCO PU-980 HPLC system. Conditions: linear gradient from 15% to 45% solvent B (solvent A, 0.1 M triethylammonium acetate (pH 7.4); solvent B, 80% acetonitrile/20% 0.1 M triethylammonium acetate (pH 7.4)).

LAP Assay on 96-Well Plates. Experiments were performed on PLL-coated black plates (clear bottom) (Iwaki, Japan) at room temperature. First, LAP was added to 100 mM Tris-HCl buffer (pH 7.4). For wells with inhibitor, amastatin (10 μM) was added at the same time. After 10 min, **[Tb−13]** was added to the wells, the total volume of which was 0.2 mL. The plate was incubated for 60 to 90 min, and then the luminescence intensity (ex. 325 nm, em. 545 nm) was measured with delay and gate times of 50 μs and 2.0 ms, respectively. The excitation and emission slits were 10 nm. An assay using Leu-MCA was performed with the same procedure except for the measured wavelength (ex. 380 nm, em. 440 nm) and without a delay time.

Assay of Human Serum (Using Tb Complex). Human sera from 5 healthy volunteers (normal) and 5 patients with cancer of the hepatobiliary-pancreatic systems (patients) were diluted 10 times with Tris-HCl buffer (0.1 M, pH 7.4). Then 1 μM **[Tb−13]** was added to the solution, and a TRF measurement was performed as described above at 37 °C. Enzymatic activity was calculated from the change of luminescence over the first 10 min. 1 U was defined as the enzymatic activity that cleaved 1 μmol of **[Tb-13]** per min under the assay conditions.

Assay of Human Serum (Using a Commercially Performed Method). The following experiments were performed by SRL, Inc. (Tokyo, Japan). Briefly, the substrate l-leucyl-3,5-dibromo-4-hydroxyanilide (L-Leu-DBHA) was converted by LAP in the sample to 3,5-dibromo-4-hydroxyanilide, which was coupled to *N*-ethyl-*N*-(2-hydroxy-3-sulfopropyl)-*m*-toluidine (TOOS) by another enzyme, bilirubin oxidase (BOD), to yield a green pigment. The absorbance of the pigment at 700 nm was recorded by a Hitachi 7170 autoanalyzer as a measure of the LAP activity of the sample. Experiments were performed at 37 °C.

Supporting Information Available: Synthetic procedures of **[Ln−X]** (**Ln = Tb**, **Eu**) (**X = 1−13**); photophysical properties of the complexes; comparison of kinetic parameters; inhibition constants of LAP inhibitors; emission spectra of **[Eu−8]** and **[Eu−1]**; comparison of luminescence intensity before and after the enzymatic reaction; spectral change of **[Ln−13]** upon reaction with LAP; emission intensity of **[Ln−X]** after the addition of LAP or trypsin; HPLC chart of **[Ln−X]**; LAP reaction of **[Tb−13]** on 96-well plate; limit of detection of LAP using two probes; correlation between LAP activity measured by the two methods; serum LAP assay in the presence of amastatin; list of authors of ref 19b. This material is available free of charge via the Internet at http://pubs.acs.org.

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