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A Time-Resolved Fluorescence Probe for Dipeptidyl Peptidase 4 and Its Application in Inhibitor Screening

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Abstract: The prevalence of type 2 diabetes is increasing dramatically throughout the world. Recently, dipeptidyl peptidase 4 (DPP4) was identified as a potential antidiabetes target. Many DPP4 inhibitors, such as sitagliptin and vildagliptin, have been developed and marketed, but superior therapeutic agents are still required. Therefore, we have developed new methodology for screening of DPP4 inhibitors. Absorption-based measurements with *para*-nitroaniline or fluorescence-based measurements with the coumarin derivative 7-amino-4-methylcoumarin are often used for the screening of protease inhibitors, including DPP4 inhibitors, but these strategies are not sufficiently sensitive because of interfering background absorption and fluorescence, thus giving rise to many false-positive and false-negative results. Therefore, we have designed and synthesised a novel DPP4 probe (Gly-Pro-BCD-Tb; Gly = glycine, Pro = proline, andBCD defines the backbone of the probe comprising an aniline derivative as on/off switch, a 7-amino-4-methyl-2(1*H*)-quinolinone (cs-124) as antenna moiety, and a diethylenetriamine-*N*,*N*,*N*',*N*'',*N*''-pentaacetic acid (DTPA) as chelator moiety, Tb = terbium) for time-resolved fluorescence (TRF) measurements. TRF measurements with Gly-Pro-BCD-Tb showed high sensitivity and reliability in the inhibitory assay relative to Gly-Pro-MCA (MCA = 4-methylcoumarin-7-amide), a conventional fluorescence probe for DPP4. Further, we employed our probe for high-throughput DPP4 inhibitor screening with 3841 randomly selected compounds and found that epibestatin, an epimer of bestatin (a well-known anticancer drug and general aminopeptidase inhibitor), showed dose-dependent DPP4 inhibitory activity. Interestingly, bestatin did not exhibit DPP4 inhibitory activity. We believe that this screening system will be useful for the discovery of DPP4 inhibitors with novel structural scaffolds.

Keywords: diabetes · fluorescence spectroscopy · high-throughput screening · lanthanides · luminescence · proteases

Introduction

An estimated 246 million people worldwide have type 2 diabetes, and this number is predicted to grow to 366 million by 2030.[1] Treatment of type 2 diabetes generally begins with oral antidiabetic agents, such as biguanides and sulfonylureas,[2] but these agents have serious adverse effects, such as lactic acidosis and hypoglycemia. So, a novel therapeutic strategy for type 2 diabetes is desired. Incretin hormones, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulinotropic polypeptide, are candidates for the treatment of type 2 diabetes.[3] These hormones are released from the gut in response to food uptake and play an important role in glucose homeostasis. For example, GLP-1 stimulates insulin biosynthesis and secretion in pancreatic β-cells and also inhibits glucagon release from pancreatic α-cells. Moreover, GLP-1 regulates insulin in a strictly glucose-dependent manner, that is, it is ineffective during

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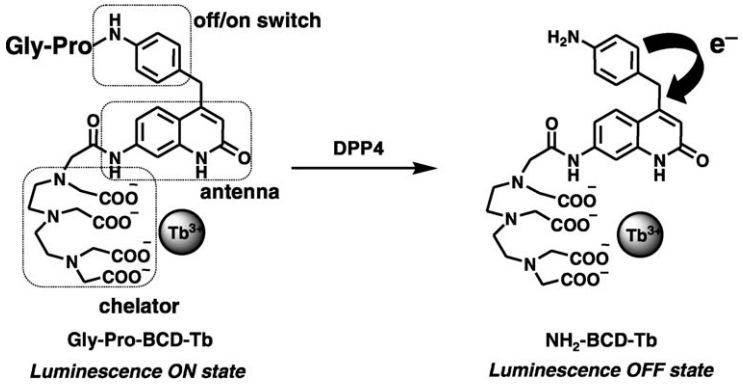
hypoglycemia. Therefore, GLP-1 therapy may be attractive for treating diabetes without risk of hypoglycemia. However, active GLP-1(7–36) amide is promptly hydrolysed in the blood through the action of dipeptidyl peptidase 4 (DPP4), a serine protease. DPP4 cleaves *N*-terminal Xxx-Pro dipeptides from polypeptides with a proline (Pro) residue at the penultimate position and generates an inactive GLP-1(9–36) amide. Therefore, DPP4 inhibitors are expected to increase the half-life of active GLP-1 in the blood and to prolong the beneficial effects of incretin hormones. So far, several DPP4 inhibitors have been reported,[5–8] and some have already been marketed as therapeutic agents for type 2 diabetes. For example, sitagliptin (Januvia, Merck),[5] vildagliptin (Galvus, Novartis),[6] and saxagliptin (Onglyza, Bristol–Myers Squibb/AstraZeneca),[7] which lack the α-amino acid moiety of early DPP4 inhibitors, are available.

Methods to screen DPP4 inhibitors include absorption measurements based on the liberation of *para*-nitroaniline[6,7,9] and fluorescence measurements with coumarin derivatives.[5] HPLC analysis of inactive GLP-1 generated by DPP4 is also performed as a gold-standard method because it employs the native substrate and reaction. However, a sensitive high-throughput method is still needed to find potent and specific inhibitors by screening large numbers of compounds with low levels of false-positive and false-negative results. Generally, background fluorescence derived from the microplates and scattered excitation light limit the sensitivity in inhibitor screening with 96- or 384-well microplates. These phenomena are especially problematic when fluorophores such as coumarin derivatives are used because coumarin derivatives are excited by short-wavelength light. However, coumarin derivatives are often used as fluorescent substrates for proteases. Luminescent lanthanide complexes offer an attractive solution to these problems. If an appropriate chelator is used, a lanthanide (especially Tb^{3+} or Eu^{3+} ions) complex can show strong luminescence with an extraordinarily long luminescence lifetime on the order of milliseconds in contrast to typical organic fluorophores, which have a short fluorescence lifetime in the nanosecond region. By taking advantage of this feature, the influence of short-lived background fluorescence and scattered light can be reduced to a negligible level by means of time-resolved fluorescence (TRF) measurements (see Figure S1 in the Supporting Information). In TRF measurements, the fluorescence signal is collected for a certain gate-time after an appropriate delay following a pulsed excitation. By employing this method, it is easy to distinguish long-lived lanthanide luminescence from other signals. Furthermore, lanthanide complexes have a large Stokes shift (>200 nm), which also helps to reduce background signals.[10,11]

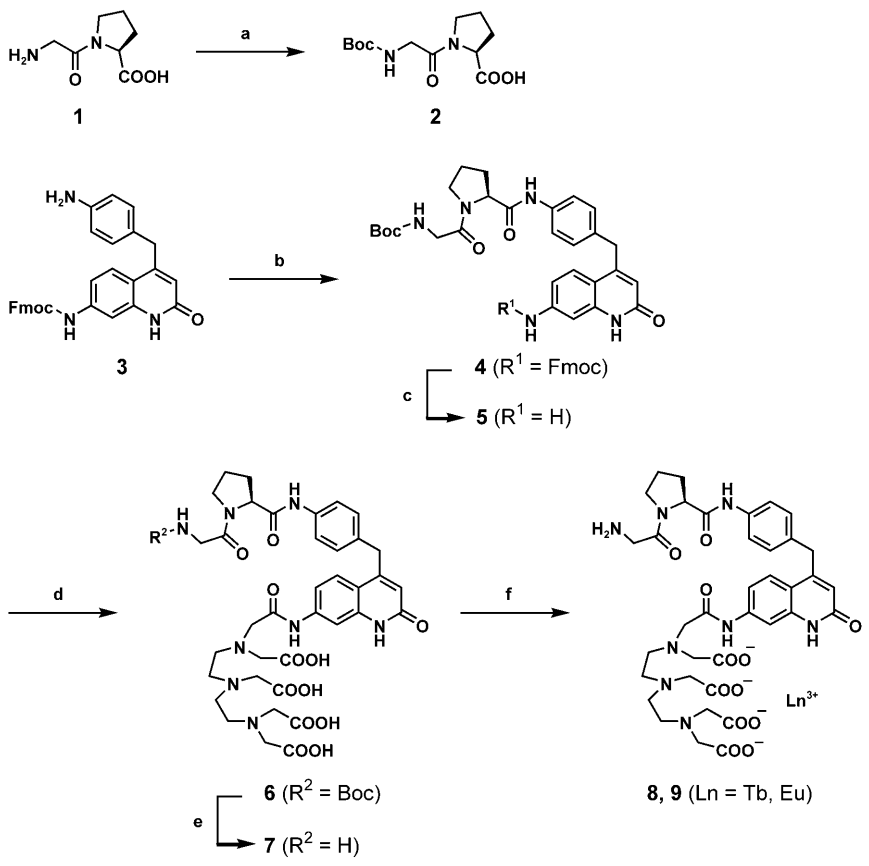
Various protease activity assays based on TRF measurements have been reported, such as time-resolved Förster resonance energy transfer (TR-FRET) by Karvinen et al.[12] and a luminescence off/on strategy that employed a simple aniline structure by Mizukami et al.[13] However, there are problems with these strategies. In the case of the former, the synthesis of the probe is very laborious; in the case of the latter, the excitation wavelength is too short for screening because of the use of the aniline moiety as an antenna. Thus, these methods are not suitable for simple and sensitive screening. Therefore, we focused on a simple quenching principle of photo-induced electron transfer (PeT)[11] previously reported by our group. In that study, we reported the measurement of leucine aminopeptidase (LAP) activity with a TRF probe. However, high-throughput screening (HTS) with a TRF probe based on the PeT mechanism has not been reported yet. We expected that HTS with a TRF probe would offer high sensitivity and good reliability. Herein, we report the development of a DPP4 probe based on the PeT mechanism and its application to HTS for DPP4 inhibitors. We found an interesting DPP4 inhibitor among 3841 samples screened with this HTS.

Results and Discussion

Probe design and strategy: We developed a lanthanide-based luminescence probe for DPP4 based on the PeT mechanism (Scheme 1). The probe consists of three moieties, which are an antenna, an off/on switch, and a chelator. The substrate peptide sequence of DPP4 glycylproline was attached to the amino group of the luminescence off/on switch moiety. Lanthanide metal ions Tb^{3+} and Eu^{3+} were selected as they show long-lived luminescence, that is, after an enzymatic reaction with DPP4 glycylproline amide is converted into the aniline derivative NH_2-BCD-Tb, which has a luminescence intensity that is greatly decreased through the a-PeT (acceptor-excited PeT) mechanism.[10] The probes Gly-Pro-BCD-Tb and Gly-Pro-BCD-Eu (where BCD defines the backbone of the probe comprising an aniline derivative as on/off switch, a 7-amino-4-methyl-2(1*H*)-quinolinone (cs-124) as antenna moiety, and a diethylenetriamine-*N*,*N*,*N*',*N*'',*N*''-pentaacetic acid (DTPA) as chelator moiety) were synthesised in six steps (Scheme 2). A standard substrate of DPP4 is Gly-Pro-MCA, which is a conven-



Scheme 1. Schematic representation of the DPP4 probe. Off/on switch moiety: aniline derivative; antenna moiety: 7-amino-4-methyl-2(1*H*)-quinolinone (cs124); chelator moiety: diethylenetriamine-*N*,*N*,*N*',*N*'',*N*''-pentaacetic acid (DTPA). Gly = glycine, Pro = proline.



Scheme 2. Synthesis of Gly-Pro-BCD-Tb and Gly-Pro-BCD-Eu. Reagents and conditions: a) Boc_2O, NEt_3, EtOH, 60°C→RT (quant. yield); b) **2**, HOBT, HBTU, DIEA, DMF, RT (43%); c) piperidine, DMF, RT (93%); d) DTPA bisanhydride, DIEA, DMF, RT (50%); e) CH_2Cl_2/TFA, 0°C (83%); f) $LnCl_3{\cdot}6H_2O$, MeOH, RT (quant. yield). Boc = *tert*-butoxycarbonyl, DIEA = *N*,*N*-diisopropylethylamine, Fmoc = 9-fluorenylmethoxycarbonyl, HBTU = *O*-benzotriazole-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate , HOBT = 1-hydroxybenzotriazole, TFA = trifluoroacetic acid.

tional coumarin-based substrate, and was also synthesised (Scheme 3).

Photochemical properties of the probes: The enzymatic reactions of Gly-Pro-BCD-Tb and Gly-Pro-BCD-Eu with DPP4 were monitored. Both probes were good substrates for DPP4 and large changes in the luminescence intensity were observed after the enzymatic reaction (Figure 1A–C). We adopted Gly-Pro-BCD-Tb as a DPP4 substrate in the following experiments because it showed a greater change in luminescence intensity ($FI_{0\,min}$/ $FI_{30\,min}$ = 55.3:1) than Gly-Pro-BCD-Eu ($FI_{0\,min}$/$FI_{30\,min}$ = 19.5:1). We also confirmed that Gly-Pro-MCA showed an increase in fluorescence after the enzymatic reaction ($FI_{30\,min}$/ $FI_{0\,min}$ = 12.5:1, data not shown).

The kinetic parameters of Gly-Pro-BCD-Tb and Gly-Pro-MCA were determined from Michaelis–Menten plots (Table 1). The K_m and k_{cat} values of Gly-Pro-BCD-Tb were 33.6 μM and 28.3 s^{-1}, respectively. The k_{cat}/K_m value was 0.85 $\mu M^{-1}s^{-1}$. The K_m, k_{cat}, and k_{cat}/K_m values for Gly-Pro-BCD-Tb were identical with those values of Gly-Pro-MCA (34.0 μM, 25.9 s^{-1}, and 0.76 $\mu M^{-1}s^{-1}$, respectively). These results indicate that the rates of the enzymatic reaction of Gly-Pro-BCD-Tb and Gly-Pro-MCA are almost the same when the substrate concentration is sufficiently low. Thus, Gly-Pro-BCD-Tb is a good substrate for DPP4 from the viewpoint of kinetic parameters.

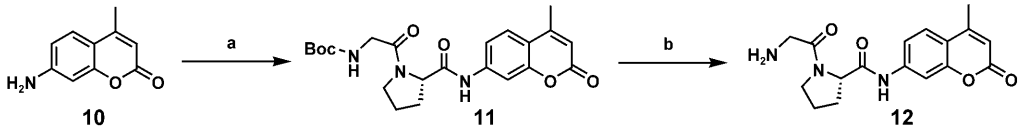
The conversion of Gly-Pro-BCD-Tb into NH_2-BCD-Tb was confirmed by HPLC analysis (Figure 1D). This outcome indicates that the enzymatic reaction proceeded as expected (Figure 1D).

Table 1. Kinetic parameters of Gly-Pro-BCD-Tb and Gly-Pro-MCA.[a]

Substrate	Gly-Pro-BCD-Tb	Gly-Pro-MCA
K_m [μM]	33.6	34.0
V_{max} [μM min^{-1}]	5.9	5.4
k_{cat} [s^{-1}]	28.3	25.9
k_{cat}/K_m [$\mu M^{-1}s^{-1}$]	0.85	0.76

[a] All data were obtained at pH 8.0 (50 mM HEPES buffer).

Comparison of detection sensitivity: Next, we compared the sensitivity of Gly-Pro-BCD-Tb and Gly-Pro-MCA (10 and 100 nM, respectively) by using a microplate reader (Figure 2). We measured the inhibitory activity of diprotin A,[14] a conventional DPP4 inhibitor, with the two above



Scheme 3. Synthesis of Gly-Pro-MCA. Reagents and conditions: a) **2**, HATU, DIEA, DMF, RT (crude product); b) CH_2Cl_2/TFA, 0°C (8% over 2 steps). MCA = 4-methylcoumarin-7-amide, HATU = *O*-(7-azabenzotriazol-1-yl)tetramethyluronium hexafluorophosphate.

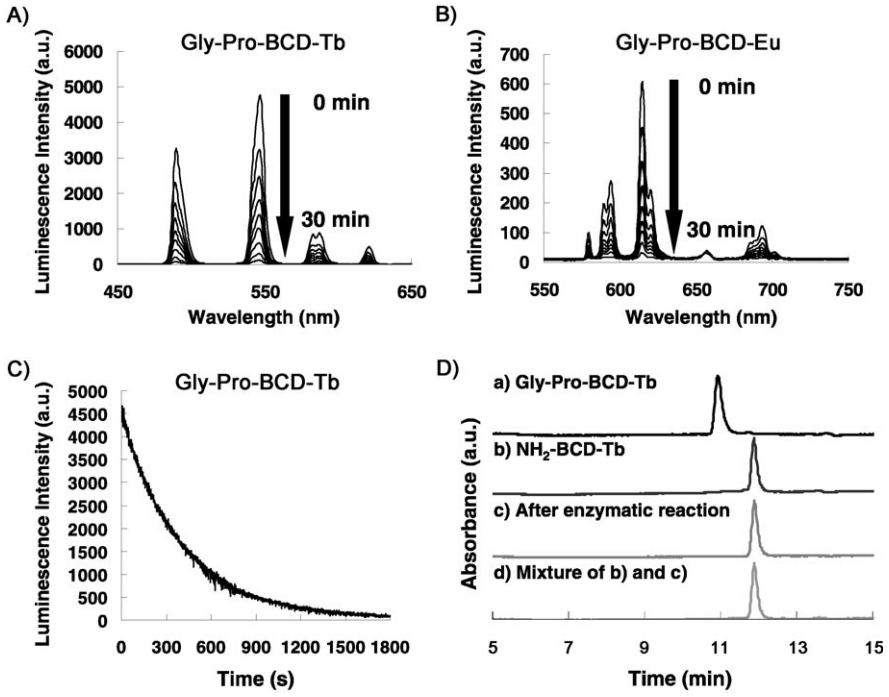


Figure 1. Fluorescence spectra of 5 μM solution of Gly-Pro-BCD-Tb (A) and Gly-Pro-BCD-Eu (B) at 0, 2, 4, 6, 8, 10, 14, 20, and 30 min after the addition of 7 nM (final) of DPP4. The reactions were performed in 50 mM HEPES buffer (pH 8.0) containing 1% dimethyl sulfoxide (DMSO) at room temperature. Excitation wavelength was $\lambda = 330$ nm. C) Time course of the luminescence intensity of Gly-Pro-BCD-Tb with DPP4 (7 nM). Excitation and emission wavelengths were $\lambda = 330$ and 546 nm, respectively. D) HPLC charts of the enzymatic reaction of Gly-Pro-BCD-Tb to afford NH_2-BCD-Tb: a) A solution of Gly-Pro-BCD-Tb, b) a solution of NH_2-BCD-Tb, c) reaction mixture after the enzymatic reaction, d) mixed solution of (b) and (c). The chromatogram was monitored at $\lambda = 330$ nm for all the spectra. Stationary phase: reversed-phase column (Inertsil ODS-3 4.6×250 mm; GL Sciences). HPLC analysis: eluent A (100 mM triethylammonium acetate (TEAA) buffer) and eluent B (acetonitrile/H_2O 80:20); A/B 90:10→70:30 (30 min).

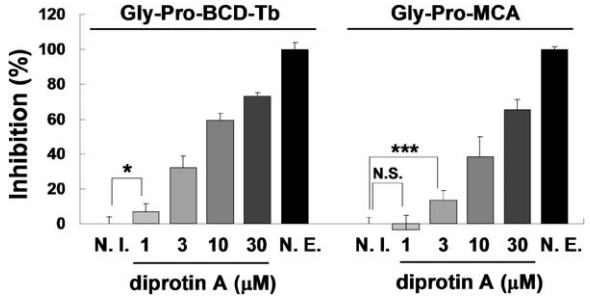


Figure 2. A comparison of sensitivity between Gly-Pro-BCD-Tb (10 nM) and Gly-Pro-MCA (100 nM). The indicated concentration of diprotin A was added to each sample. The concentration of DPP4 was 1 nM. TRF measurements were performed with a delay time of 0.05 ms and a gate-time of 0.95 ms. Excitation wavelengths were $\lambda = 330$ and 380 nm for Gly-Pro-BCD-Tb and Gly-Pro-MCA, respectively, and the luminescence intensity was measured at $\lambda = 546$ and 440 nm for Gly-Pro-BCD-Tb and Gly-Pro-MCA, respectively. The measurements were performed with a microplate reader. * indicates $P < 0.05$, *** indicates $P < 0.001$, NS = not significant, N.I. = no inhibitor, N.E. = no enzyme. The data are the mean ± SD ($n = 6$).

sensor probes. TRF measurements with Gly-Pro-BCD-Tb detected inhibition by 1 μM diprotin A, whereas measurements with Gly-Pro-MCA required a concentration of 3 μM diprotin A before inhibitory activity could be observed. Thus, we could detect even weak inhibitors, which are often seen in initial screening, with Gly-Pro-BCD-Tb.

Further evaluation of Gly-Pro-BCD-Tb: We next examined the usefulness of Gly-Pro-BCD-Tb in a HTS system relative to Gly-Pro-MCA. First, 1 and 10 nM Gly-Pro-BCD-Tb were sufficient for the TRF measurements with a microplate reader, whereas 100 nM Gly-Pro-MCA was needed for general fluorescence measurements. This result is due to the low background luminescence of TRF measurements and relatively high background fluorescence of ordinary fluorescence measurement, as already noted. The difference in IC_{50} values of diprotin A and sitagliptin (a novel DPP4 inhibitor)[5] are shown in Figure 3 and Table 2, as determined by using 1 and 10 nM Gly-Pro-BCD-Tb and 100 nM Gly-Pro-MCA. The IC_{50} values depend on the affinity and the concentration of each probe (Figure 3D), and a lower concentration of probe gives a smaller IC_{50} value (Figure 3A,B). In inhibitor screening, a one-point measurement (i.e., the concentration of all the samples is fixed, e.g., at 10 μM) is often used, and it is important that the IC_{50} value should be small. For example, if the IC_{50} value is one order larger, it may not be possible to estimate the inhibitory activity of a sample accurately (Figure 3C). For screening, it is important to identify samples even with weak inhibitory activity. Thus, Gly-Pro-BCD-Tb is superior to Gly-Pro-MCA.

Table 2. IC_{50} values of sitagliptin and diprotin A with Gly-Pro-BCD-Tb and Gly-Pro-MCA.[a]

	Sitagliptin [nM]	Diprotin A [μM]
Gly-Pro-BCD-Tb (1 nM)	2.0	–[b]
Gly-Pro-BCD-Tb (10 nM)	6.0	9.4
Gly-Pro-MCA (100 nM)	15.5	34.1

[a] All data were obtained at pH 8.0 (50 mM HEPES buffer). [b] Not determined.

Next, the inhibitory activity of sitagliptin was measured with Gly-Pro-BCD-Tb and Gly-Pro-MCA in the presence of fluorescent contaminants (Figure 4). Fluorescent contaminants were selected from screening samples as inactive compounds that showed strong absorption and fluorescence

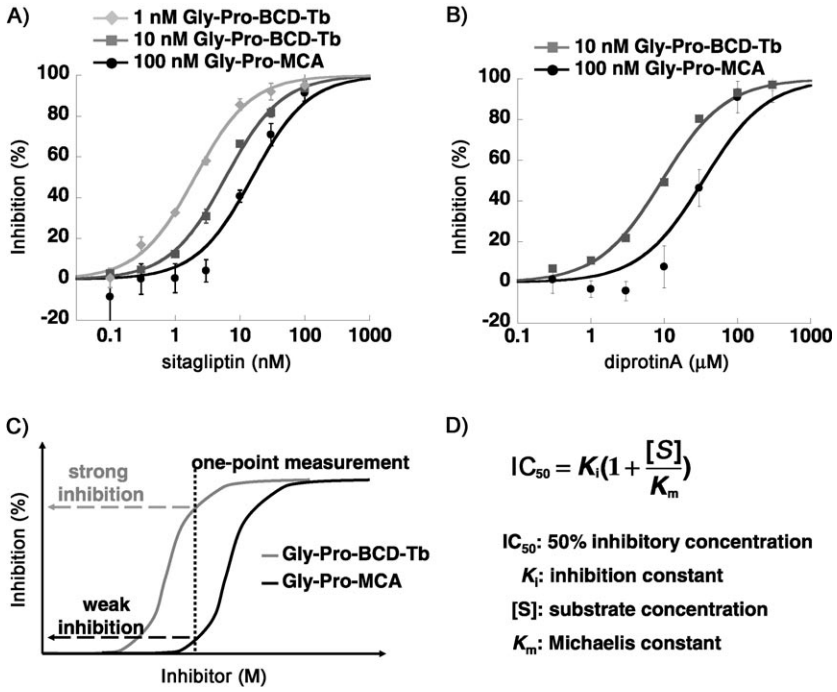


Figure 3. Inhibition curve of sitagliptin (A) and diprotin A (B). Diamond: 1 nM Gly-Pro-BCD-Tb, square: 10 nM Gly-Pro-BCD-Tb, circle: 100 nM Gly-Pro-MCA. Each data point represents the mean ± SD ($n=6$). C) Difference in inhibition (%) between Gly-Pro-BCD-Tb and Gly-Pro-MCA at a one-point measurement. D) Equation for the IC_{50} value.

overlapping with those of Gly-Pro-BCD-Tb or Gly-Pro-MCA. (The absorption and fluorescence spectra and the structure of each sample are shown in Figure S3 in the Supporting Information.) When the inhibitory activity of sitagliptin was measured in the presence of a contaminant, the inhibitory activity was successfully detected with Gly-Pro-BCD-Tb. In contrast, Gly-Pro-MCA was ineffective because of the very strong background fluorescence. In the case of Gly-Pro-BCD-Tb, the measured IC_{50} value was almost the same in the presence of samples 1–3 as fluorescent contaminants, that is, the inhibitory activity could be measured accurately with Gly-Pro-BCD-Tb, even in the presence of fluorescent contaminants. This result indicates that we can precisely evaluate the inhibitory activities of screening samples with Gly-Pro-BCD-Tb, even if the samples are strongly fluorescent.

DPP4 inhibitor screening: From above results, we considered that Gly-Pro-BCD-Tb is superior to Gly-Pro-MCA for high-sensitivity screening of DPP4 inhibitors. We performed screening of DPP4 inhibitors among 3841 randomly selected samples (Figure 5A). Among the 3841 samples (each microplate contains 80 samples), there were seven that showed more than 50% inhibition at 10 μM and several samples also exhibited about 40% inhibition at 10 μM. We obtained titration curves of the above seven compounds for the inhibition of DPP4. Only one sample showed dose-dependent inhibitory activity (Figure 5B). This sample was epibestatin ($IC_{50}=$ 17 μM), which is an epimer of the anticancer drug bestatin. Interestingly, bestatin itself did not show any inhibitory activity (Figure 5C). To our knowledge, there are no reported examples of epibestatin that have shown a stronger inhibitory activity than bestatin.[15,16] For example, bestatin is a comparatively strong inhibitor of general aminopeptidases, such as leucine aminopeptidase (LAP; $IC_{50}=$ 0.032 μM), whereas epibestatin is a weak inhibitor ($IC_{50}=$ 24 μM).[16] However, DPP4 is a serine aminopeptidase that recognises substrates that contain a proline residue, and we speculate that the unique structural characteristics of the proline residue account for this apparent anomaly. Among samples with similar chemical structures to epibestatin, there were none that exhibited a higher activity than epibestatin (data not shown). Furthermore, when we compared the assay results that used Gly-Pro-BCD-Tb and Gly-Pro-MCA, epibestatin was not detected with Gly-Pro-MCA, whereas another sample (square point in Figure 5D) was detected as a strong inhibitor. However, further examination showed that this strong inhibitor was a false-positive result. (The structure and photochemical properties of this inhibitor, a 4-amide-1,8-naphthalimide derivative, are shown in Figure S4 in the Supporting Information.) Naphthalimides are known to be strongly fluorescent,[17] and indeed the compound showed excitation and emission absorptions at $\lambda=$ 350 and 485 nm, respectively, in 2-amino-2-(hydroxymethyl)-1,3-propanediol hydrochloride (Tris–HCl) buffer (pH 8.0). Therefore, it is considered that accurate measurements could not be carried out with Gly-Pro-MCA (Figure 5D) due to overlap of the excitation and emission wavelengths. False-positive samples were seen in other sample plates when Gly-Pro-MCA was used (not shown) but not in TRF measurements with Gly-Pro-BCD-Tb. We also examined the dose dependency of epibestatin with Gly-Pro-MCA (see Figure S5 in the Supporting Information). Marginal inhibitory activity of epibestatin (10 μM) towards DPP4 was detected. Only weak inhibitory activity was seen with Gly-Pro-MCA relative to the activity with Gly-Pro-BCD-Tb (Figure 5C). It is considered that this outcome is due to the difference in sensitivity between Gly-Pro-MCA and Gly-Pro-BCD-Tb (Figures 2 and 3). These results indicate that sensitive and reliable screening can be carried out with Gly-Pro-BCD-Tb.

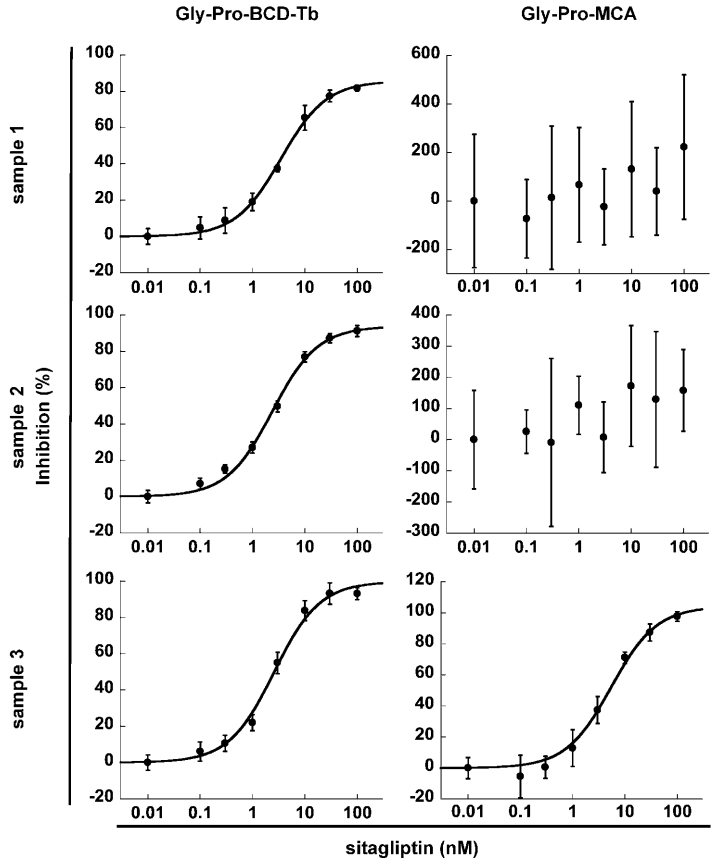


Figure 4. Inhibition curves of sitagliptin with Gly-Pro-BCD-Tb and Gly-Pro-MCA, containing 10 μM solution of samples **1** and **3** and 1 μM solution of sample **2** as contaminants (the fluorescence intensity of 10 μM sample **2** was too strong to be measured with the microplate reader). Each data point represents the mean ± SD ($n=6$).

Conclusion

We have developed the novel DPP4 probe Gly-Pro-BCD-Tb. This probe showed almost the same reactivity with DPP4 as Gly-Pro-MCA, but is superior in two respects: 1) it offers greater sensitivity, so that even weak inhibitors can be detected and 2) it is suitable for TRF measurements, which can be accurately carried out, even in the presence of strong background fluorescence and scatted light. The false-positive rate with Gly-Pro-BCD-Tb was much smaller than with Gly-Pro-MCA, as expected. We carried out high-throughput TRF-based DPP4 inhibitor screening of about 4000 samples with Gly-Pro-BCD-Tb, and epibestatin has been newly identified as a DPP4 inhibitor. Interestingly, bestatin itself was not an inhibitor. This difference in inhibitory activity against DPP4 between bestatin and epibestatin may lead to the discovery of novel scaffolds for DPP4 inhibitors. The present methodology is based on the fluorescence decrease that resulted from DPP4 activity. It is necessary to consider the possibility of false-negative results in the inhibitor screening that result from competitive chelation of the metal ion or collisional quenching. However, the chelation between DTPA and the Tb^{3+} ion is very strong ($\log K_M = 22.71$ and 22.39 for Tb and Eu, respectively),[18] and the concentration of Gly-Pro-BCD-Tb in this screening is sufficiently low. Therefore, we think that this on/off type methodology with TRF measurements is suitable for screening. The present methodology is also expected to be applicable for screening inhibitors of other proteases, if the substrate sequence of Gly-Pro-BCD-Tb is appropriately modified.

Experimental Section

General information: HBTU, HOBT, HATU, and glycylproline were purchased from Watanabe Chemical Industries, Ltd (Japan). DPP4 was purchased from R&D Systems (USA). All the other reagents and solvents were purchased from Tokyo Kasei Co., Ltd (Japan) or Wako Pure Chemical Industries, Ltd (Japan) and were of the highest grade available and used as received. The ^{1}H NMR spectra were recorded on JEOL JNM-LA300 and JEOL JNM-LA400 instruments; δ values are given in ppm from the solvent resonance as the internal standard. Data are reported as follows: chemical shift, multiplicity (s = singlet, d = doublet, t = triplet, br = broad, and m = multiplet), integration, J coupling, and assignment. Mass spectra were recorded on a JEOL JMS-T100 LC mass spectrometer. Column chromatography was performed on silica gel 60 (Kanto Chemical Co.; Tokyo, Japan) and Chromatorex-NH silica gel (Fuji Silysia Chemical; Kasugai, Japan). Purification by preparative HPLC was performed on a reverse-phase ODS column (GL Sciences, Japan; Inertsil ODS-3 10×250 mm) with 0.1% TFA in water as eluent A and 0.1% TFA in acetonitrile/water (80:20) as eluent B or 100 mM TEAA in water as eluent A and acetonitrile/water (80:20) as eluent B on a JASCO PU-2080 plus system at a flow rate of 5.0 mL min^{-1}. HPLC analyses were performed on a reversed-phase ODS column (GL Sciences; Inertsil ODS-3 4.6×250 mm) fitted onto a JASCO PU-980 plus system at a flow rate of 1.0 mL min^{-1}. UV/Vis spectra were obtained on a Shimazu UV-1600 spectrometer (Tokyo, Japan). Fluorescence spectra were obtained on a Hitachi F4500 (Tokyo, Japan). Time-resolved fluorescence assay on 96-well plates were performed using Perkin–Elmer EnVision Multilabel Plate Readers (Beaconsfield, Buckinghamshire, England).

Determination of kinetic parameters: Various concentrations of probes (Gly-Pro-BCD-Tb and Gly-Pro-MCA) were dissolved in 2-[4-(2-hydroxyethyl)piperazin-1-yl]ethanesulfonic acid (HEPES) buffer (50 mM, pH 8.0). DPP4 was added to the solution, and the fluorescence intensity was recorded continuously. The initial reaction velocity was calculated and plotted against probe concentration. The points were fitted to a Michaelis–Menten curve, and the K_m and k_{cat} values were determined by the least-squares method.

HPLC analysis: Reversed-phase HPLC analysis was performed using an Inertsil ODS-3 column (4.6×250 mm; GL Science, Japan) fitted onto a JASCO PU980 HPLC system to confirm that the enzymatic reaction had taken place. The conditions and recorded absorption wavelength are given in the figure legends.

DPP4 inhibition assay on 96-well plates: Experiments were performed on Costar 96-well half-area black plates. Gly-Pro-BCD-Tb (40 nM, 10 μL) was added to Tris–HCl buffer (50 mM, 10 μL; pH 8.0). To each well, the inhibitor (40 μM, 10 μL; 2% DMSO) was added at the same time and DPP4 (3.5 nM, 10 μL) was added. The plate was incubated at room temperature for 90 min. The fluorescence intensity ($\lambda_{ex}=330$ nm and $\lambda_{em}=545$ nm) was measured with a delay time of 0.05 ms and a gate-time of 0.95 ms. An assay with Gly-Pro-MCA was performed similarly, except for the measured wavelength ($\lambda_{ex}=355$ nm and $\lambda_{em}=460$ nm) and without a delay time.

Screening of DPP4 inhibitors: High-throughput screening was performed against a part of the chemical library (3841 compounds) of the Chemical Biology Research Initiative (The University of Tokyo). All the samples (buffer, Gly-Pro-BCD-Tb, DPP4, and candidate inhibitors (samples)) were dispensed through a dispenser. The fluorescence intensities at 0 and

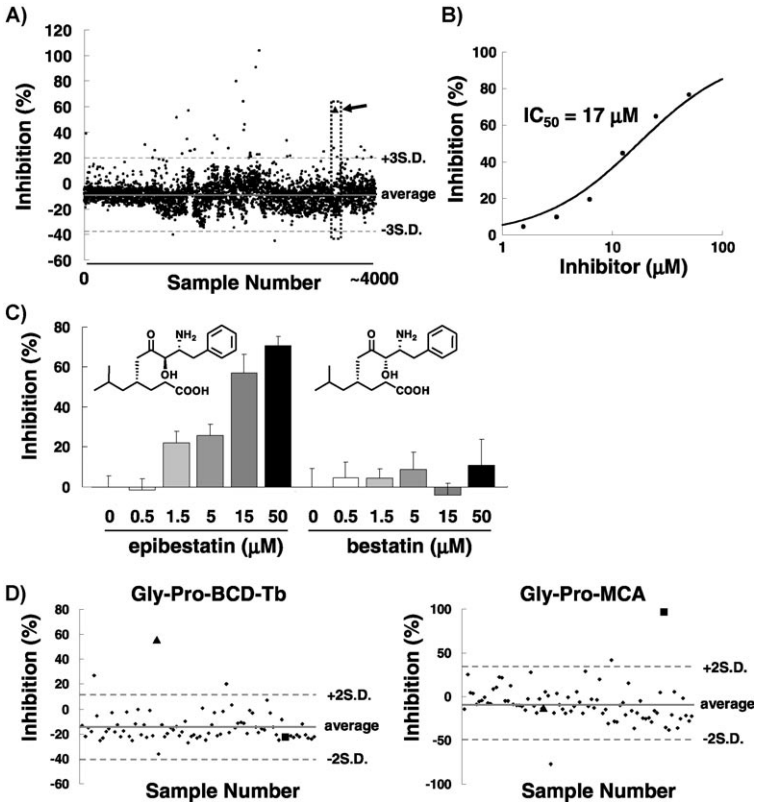


Figure 5. A) Results of the screening assay for DPP4 inhibitors. A total of 3841 samples were assayed. The large triangle and arrow indicate epibestatin and the dashed box indicates a microplate containing epibestatin. Each microplate contained 80 compounds. B) Inhibition curve of epibestatin. The calculated IC_{50} value was 17 μM. C) A comparison of DPP4 inhibitory activity of epibestatin and bestatin. The chemical structures are also shown. The data are means ± SD ($n=6$). D) Assay result of a microplate (80 samples, dashed box shown in (A)); left: Gly-Pro-BCD-Tb, right: Gly-Pro-MCA. Triangular and square points indicate epibestatin and a false-positive compound, respectively. SD = 12.9 for left, SD = 21.4 for right.

90 min were measured with a microplate reader after the addition of the enzyme. The microplates were incubated at room temperature during the measurement. The conditions were the same as described for the DPP4 inhibition assay.

Estimation of inhibition (%): All the values of inhibition were calculated in accordance with Equation (1), in which $L_{0\,min}$ = initial luminescence intensity, $L_{90\,min}$ = luminescence intensity at 90 min.

$$\text{Inhibition } (\%) = [(L_{90\,min}/L_{0\,min})_{sample} - (L_{90\,min}/L_{0\,min})_{no\ inhibitor}]/ [(L_{90\,min}/L_{0\,min})_{no\ enzyme} - (L_{90\,min}/L_{0\,min})_{no\ inhibitor}] \times 100 \quad (1)$$

Synthesis of 2: Boc_2O (253 mg, 1.16 mmol) was added to a solution of glycylproline (**1**; 100 mg, 0.58 mmol) and NEt_3 (800 μL, 5.8 mmol) in distilled EtOH (10 mL). The reaction mixture was stirred at 60 °C for 1 h and at room temperature for 3 h. The reaction mixture was evaporated, and the residue was dissolved in CH_2Cl_2, washed with 0.1 M HCl and brine, dried over Na_2SO_4, and evaporated. The residue was purified by column chromatography on silica gel to obtain **2** as a colourless solid (157 mg, 0.58 mmol, quant. yield). ^{1}H NMR (300 MHz, $CDCl_3$): $\delta = 1.44$ (s, 9H, CH_3), 1.87–2.35 (m, 4H, CH_2–CH_2), 3.40–3.70 (m, 2H, CH_2–CH_2), 3.81–4.11 (m, 2H, CH_2), 4.54–4.62 (m, 1H, CH), 9.48 ppm (br s, 1H, CONH); ^{13}C NMR (75 MHz, $CDCl_3$): $\delta = 24.5$, 28.3, 42.9, 46.2, 59.2, 79.9, 80.5, 156.0, 168.7, 174.0 ppm; HRMS (ESI^-): *m/z*: calcd for $C_{12}H_{19}N_2O_5$: 271.1294 [$M-H^-$]; found: 271.1298.

Synthesis of 4: Compound **2** (87 mg, 0.32 mmol) was added to a solution of **3** (78 mg, 0.16 mmol), HBTU (150 mg, 0.4 mmol), HOBT (61 mg, 0.4 mmol), and DIEA (120 μL, 0.64 mmol) in dehydrated DMF (5 mL). The reaction mixture was stirred at room temperature overnight under argon and evaporated. The residue was dissolved in AcOEt and the solution was washed with sodium phosphate buffer (pH 4.4), sodium phosphate buffer (pH 9.2), and brine; dried over Na_2SO_4; and evaporated. The residue was purified by column chromatography on silica gel (eluent: CH_2Cl_2/MeOH 19:1→9:1) to obtain **4** as a colourless powder (51 mg, 0.068 mmol, 43%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.41$ (s, 9H, CH_3), 1.74–2.15 (m, 4H, CH_2-CH_2), 3.40–3.60 (m, 2H, CH_2-CH_2), 3.63–3.87 (m, 2H, CH_2), 4.05 (s, 2H, CH_2), 4.31 (t, 1H, $J = 6.6$ Hz, Ar–CH), 4.37–4.43 (m, 1H, CH), 4.48 (d, 2H, $J = 6.6$ Hz, CH_2), 6.10 (s, 1H, ArH), 6.74–6.85 (m, 1H, CONH), 7.11 (d, 1H, $J = 8.1$ Hz, ArH), 7.20 (d, 2H, $J = 8.4$ Hz, ArH), 7.30–7.46 (m, 4H, ArH), 7.51 (d, 2H, $J = 8.4$ Hz, ArH), 7.58–7.67 (m, 2H, ArH), 7.75 (d, 2H, $J = 7.3$ Hz, ArH), 7.90 (d, 2H, $J = 8.1$ Hz, ArH), 9.92 (s, 1H, CONH), 10.02 (s, 1H, CONH), 11.60 ppm (s, 1H, CONH); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 24.4$, 28.2, 29.2, 36.7, 36.8, 42.4, 46.5, 60.2, 65.8, 77.9, 103.5, 112.8, 114.1, 119.1, 119.3, 120.2, 125.1, 125.5, 127.1, 127.7, 129.0, 129.1, 133.0, 137.4, 140.0, 140.8, 140.8, 143.7, 150.3, 153.2, 155.8, 162.0, 167.5 ppm; HRMS (ESI^+): *m/z*: calcd for $C_{43}H_{43}N_5NaO_7$: 764.3079 [$M+Na^+$]; found: 764.3060.

Synthesis of 5: Piperidine (200 μL) was added to a solution of **4** (23 mg, 0.031 mmol) in dehydrated DMF (4 mL). The reaction mixture was stirred at room temperature for 1 h and evaporated. The residue was purified by column chromatography on NH silica gel (eluent: CH_2Cl_2/MeOH 19:1→9:1) to obtain **5** as a colourless powder (15 mg, 0.029 mmol, 93%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.43$ (s, 9H, CH_3), 1.80–2.20 (m, 4H, CH_2–CH_2), 3.46–3.63 (m, 2H, CH_2–CH_2), 3.70–3.96 (m, 2H, CH_2), 4.03 (s, 2H, CH_2), 4.44–4.52 (m, 1H, CH), 5.82 (s, 2H, NH_2), 5.90 (s, 1H, ArH), 6.40–6.48 (m, 2H, ArH), 6.82–6.93 (m, 1H, CONH), 7.24 (d, 2H, $J = 8.8$ Hz, ArH), 7.44 (d, 1H, $J = 8.8$ Hz, ArH), 7.57 (d, 2H, $J = 8.8$ Hz, ArH), 9.99 (s, 1H, CONH), 11.27 ppm (s, 1H, CONH); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 24.4$, 28.2, 29.3, 36.9, 42.4, 45.8, 60.2, 77.9, 96.8, 109.4, 110.4, 115.0, 119.3, 125.7, 129.0, 129.1, 133.5, 137.3, 141.2, 150.6, 150.9, 155.8, 162.4, 167.5 ppm; HRMS (ESI^+): *m/z*: calcd for $C_{28}H_{34}N_5O_5$: 520.2560 [$M+H^+$]; found: 520.2551.

Synthesis of 6: DTPA (20 mg, 0.056 mmol) was added to a solution of **5** (15 mg, 0.029 mmol) and DIEA (15 μL, 0.084 mmol) in dehydrated DMF (5 mL). The reaction mixture was stirred at 0 °C under argon and warmed to room temperature overnight. H_2O (2 mL) was added to quench the reaction, and the reaction mixture was evaporated. The residue was purified by reverse-phase HPLC to afford **6** as a colourless solid (13 mg, 0.015 mmol, 50%). ^{1}H NMR (300 MHz, [D$_6$]DMSO): $\delta = 1.39$ (s, 9H, CH_3), 1.80–2.18 (m, 4H, CH_2-CH_2), 2.82–3.10 (m, 8H, CH_2), 3.31–3.63 (m, 12H, CH_2), 3.66–3.92 (m, 2H, CH_2), 4.10 (s, 2H, CH_2), 4.40–4.48 (m, 1H, CH), 6.13 (s, 1H, ArH), 6.79–6.90 (m, 1H, CONH), 7.24 (d, 2H, $J = 8.4$ Hz, ArH), 7.39 (d, 1H, $J = 8.8$ Hz, ArH), 7.54 (d, 2H, $J = 8.4$ Hz, ArH), 7.67 (d, 1H, $J = 8.8$ Hz, ArH), 8.02 (s, 1H, ArH), 9.98 (s, 1H, CONH), 10.60 (br, 1H, CONH), 11.71 ppm (s, 1H, CONH); ^{13}C NMR (100 MHz, [D$_6$]DMSO): $\delta = 24.4$, 28.2, 29.3, 36.7, 42.4, 45.8, 50.2, 50.9, 50.9, 51.9, 55.0, 58.2, 59.3, 60.3, 77.9, 78.0, 104.8, 113.9, 114.7, 119.4, 119.7,

125.3, 129.1, 133.0, 137.4, 139.8, 140.4, 150.4, 155.8, 162.0, 167.6, 169.6, 169.9, 170.4, 172.6, 172.9 ppm; HRMS (ESI^-): *m/z*: calcd for $C_{42}H_{53}N_8O_{14}$: 893.3681 [$M-H^-$]; found: 893.3642.

Synthesis of 7: TFA (2 mL) was added dropwise at 0 °C to a solution of **6** (13 mg, 0.015 mmol) in CH_2Cl_2 (3 mL). The reaction mixture was stirred at 0 °C for 2 h and evaporated. The residue was purified by reverse-phase HPLC to obtain **7** as a colourless solid (10 mg, 0.012 mmol, 83 %). ^1H NMR (300 MHz, [D_6]DMSO): $\delta = 1.75–2.16$ (m, 4H, $CH_2–CH_2$), 2.74–3.60 (m, 20H, CH_2), 3.74 (s, 2H, CH_2), 4.05 (s, 2H, CH_2), 4.39–4.45 (m, 1H, CH), 6.19 (s, 1H, ArH), 7.19 (d, 2H, $J = 8.0$ Hz, ArH), 7.33 (d, 1H, $J = 8.8$ Hz, ArH), 7.44–7.61 (m, 3H, ArH), 8.00 (s, 1H, ArH), 10.09 (s, 1H, CONH), 10.80 (br, 1H, CONH), 11.67 ppm (br, 1H, CONH); ^{13}C NMR (100 MHz, [D_6]DMSO): $\delta = 22.1$, 24.2, 29.4, 37.0, 45.8, 49.8, 51.3, 51.5, 51.9, 54.7, 57.6, 58.4, 59.9, 60.3, 104.8, 114.1, 114.4, 119.4, 119.7, 125.3, 128.9, 133.4, 133.8, 137.2, 139.9, 140.5, 150.0, 162.0, 169.4, 170.0, 170.1, 173.2, 174.1 ppm; HRMS (ESI^-): *m/z*: calcd for $C_{37}H_{45}N_8O_{12}$: 793.3157 [M-H^-]; found: 793.3123.

Synthesis of Gly-Pro-BCD-Ln complexes 8 and 9: $TbCl_3 \cdot 6H_2O$ (2.4 mg, 0.007 mmol) or $EuCl_3 \cdot 6H_2O$ (2.4 mg, 0.007 mmol) were added to a solution of **7** (5 mg, 0.006 mmol) in MeOH (3 mL). The reaction mixture was stirred at room temperature for 1 h and evaporated. The residue was dissolved in H_2O and the solution was loaded on a C18 Sep-Pak cartridge (eluent: $H_2O \rightarrow$MeOH) to remove excess metal. The eluent was collected and evaporated to afford **8** (5.7 mg, 0.006 mmol, quant. yield) and **9** (5.7 mg, 0.006 mmol, quant. yield) as colourless solids. **8**: HRMS (ESI^+): *m/z*: calcd for $C_{37}H_{43}N_8NaO_{12}Tb$: 973.2152 [$M$+$Na^+$]; found: 973.2157. **9**: HRMS (ESI^+): *m/z*: calcd for $C_{37}H_{43}N_8NaO_{12}Eu$: 945.2291 [$M$+$Na^+$]; found: 945.2243.

Synthesis of Gly-Pro-MCA 12: Compound **2** (300 mg, 1.1 mmol) was added to a solution of **10** (100 mg, 0.57 mmol), HATU (324 mg, 1.14 mmol), and DIEA (300 μL, 1.7 mmol) in dehydrated DMF (10 mL). The reaction mixture was stirred at room temperature over night under argon and evaporated. The residue was dissolved in CH_2Cl_2, washed with 0.1 M HCl, dried over Na_2SO_4, and evaporated. The residue was partly purified by column chromatography on silica gel (eluent: CH_2Cl_2/MeOH 19:1→9:1) to afford **11** as a crude product (35 mg). TFA (5 mL) was added at 0 °C to a solution of **11** (35 mg) in CH_2Cl_2 (5 mL). The reaction mixture was stirred at 0 °C for 2 h and evaporated. The residue was purified by column chromatography on NH silica gel (eluent: CH_2Cl_2/MeOH 19:1→93:7) to obtain **12** as a colourless solid (5 mg, 0.046 mmol, 8 %). ^1H NMR (300 MHz, [D_6]DMSO): $\delta = 1.75–2.21$ (m, 4H, $CH_2–CH_2$), 2.39 (s, 3H, Ar–CH_3), 3.15–3.72 (m, 6H, NH_2, CH_2), 4.38–4.54 (m, 1H, CH), 6.26 (s, 1H, ArH), 7.47 (dd, 1H, $J = 1.5$, 8.8 Hz, ArH), 7.71 (d, 1H, $J = 8.8$ Hz, ArH), 7.75 (d, 1H, $J = 1.5$ Hz, ArH), 10.47 ppm (s, 1H, CONH); ^{13}C NMR (100 MHz, [D_6]DMSO): $\delta = 18.0$, 24.4, 29.3, 43.5, 45.6, 60.3, 105.6, 112.3, 115.0, 115.2, 125.9, 142.4, 153.2, 153.7, 160.1, 171.4, 171.5 ppm; HRMS (ESI^+): *m/z*: calcd for $C_{17}H_{19}N_3NaO_4$: 352.1273 [M+Na^+]; found: 352.1237.

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