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for

Pyrene Excimer Based Ratiometric Detection of a Tetra-Aspartate Tag Fused Protein Using Zn^{II}-DpaTyr Probe

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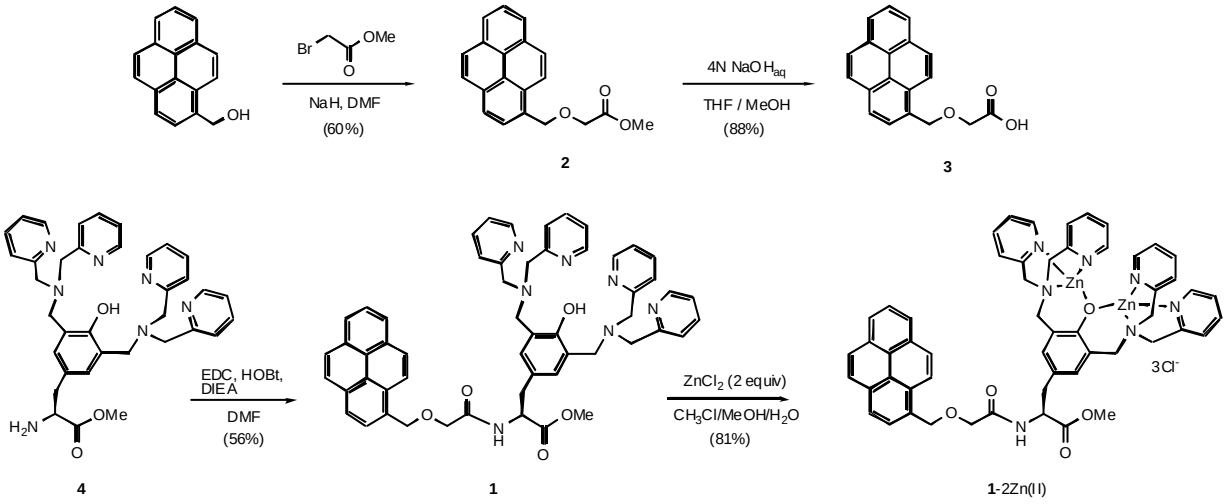
General methods

Unless otherwise noted, all chemical reagents and proteins were purchased from commercial suppliers and used without further purification. ^{1}H NMR spectra were recorded using a JNM-EX400 (JEOL, 400 MHz) spectrometer and the chemical shifts (δ, ppm) are referenced to the respective solvent. FAB mass spectra were recorded by using a QP5050A (Shimadzu).

Syntheses and compound characterization



Scheme S1 Synthesis of **1**-2Zn(II).

1-Pyrenylmethoxy acetic acid methylester (2): A mixture of 1-pyrenemethanol (400 mg, 1.72 mmol) and sodium hydride (60% oil dispersion, 140 mg, 3.44 mmol) in dry DMF (10 mL) was stirred at room temperature for 1 h. Methyl bromoacetate (0.2 mL, 2.23 mmol) was added, and the mixture was stirred for 30 min in an ice bath. After careful quenching with MeOH, the resulting mixture was poured into water and extracted with ethyl acetate (x 2). The combined organic layars were washed with saturated $NaHCO_3$ and brine followed by drying over Na_2SO_4. After removal of the solution in vacuo, and the residue was purified by column chromatography (SiO_2, CH_2Cl_2/hexane = 4:1 (v/v)) to give **2** (312 mg, 60%) as a colorless powder. ^{1}H NMR (400 MHz, $CDCl_3$): *d* 8.35 – 7.85 (9H, m), 5.19 (2H, s), 4.10 (2H, s), 3.69 (3H, s).

1-Pyrenylmethoxy acetic acid (3): A solution of 4N $NaOH_{aq}$ (8 mL) was added to a stirring solution of **2** (311 mg, 1.0 mmol) in MeOH/THF = 1:1 (v/v) (8 mL) in an ice bath, and the mixture was stirred at room temperature for 1 h. The resulting mixture was poured into 5% citric acid and extracted with $CHCl_3$ (x 2). The combined organic layers were washed with 5% citric acid and brine followed by drying over Na_2SO_4. After removal of the solution in vacuo, **3** (262 mg, 88%) was obtained as a colorless powder. ^{1}H NMR (400 MHz, $CDCl_3$): *d* 8.43 – 8.00 (9H, m), 5.39 (2H, s), 4.24 (2H, s). FAB-MS *m/e* 290 $[M + H]^+$.

Pyrene-DpaTyr (1): A mixture of **3** (49 mg, 0.17 mmol), **4**[15] (69 mg, 0.11 mmol), HOBt·H_2O (26 mg, 0.17 mmol), EDC·HCl (32 mg, 0.17 mmol) and *N,N'*-diisopropylethylamine (29 µL, 0.17 mmol) in dry DMF (2.5 mL) was stirred at room temperature for 16 h. The mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 0.1 N $NaOH_{aq}$ and brine followed by drying over Na_2SO_4. After removal of the solvent in vacuo, the residue was purified by column chromatography (SiO_2, CH_2Cl_2/MeOH/$NH_{3\,(aq)}$ = 100:0:0 → 300:10:1 (*v*/*v*/*v*)) to give **1** (56 mg, 56%) as a colorless powder. ^{1}H NMR (400 MHz, $CDCl_3$): δ 8.46 (4H, d, *J* = 4.2 Hz), 8.18 – 7.78 (9H, m), 7.50 (4H, td, *J* = 7.6, 1.6 Hz), 7.79 (4H, d, *J* = 8.0 Hz), 7.11 (1H, d, *J* = 8.4 Hz), 7.04 – 7.01 (4H, m), 6.94 (2H, s), 5.11 (2H, s), 4.85 – 4.80 (1H, m), 4.02 (2H, d, *J* = 3.6 Hz), 3.79 – 3.59 (15H, m), 3.03 – 2.97 (2H, m). FAB-HRMS *m/e* calcd for $[M]^+$ 889.3952, found 889.3952.

Pyrene-DpaTyr(Zn) (1-2Zn^{II}): To a solution of **1** (32 mg, 34 µmol) in distilled MeOH (2 mL) was added aqueous solution of $ZnCl_2$ (100 mM; 680 µL, 68 µmol), and the solution was stirred at room temperature for 1.5 h. The mixture was concentrated in

vacuo, and the residue was re-dissolved in distilled water. The solution was filtered through cellulose acetate filter (pore size ; 0.45 μm) and then lyophilized under vacuum. The obtained solid was suspended in ethyl acetate, filtered, and dried in vacuo to afford **1**-2Zn^{II} (33 mg, 81%) a colorless powder. ^{1}H NMR (400 MHz, CD_3OD): *d* 9.02 (2H, br), 8.58 – 8.17 (9H, m), 8.03 (3H, br), 7.54 – 7.36 (6H, m), 7.01 (3H, br), 6.65 (3H, br), 6.13 (2H, s), 5.44 (1H, d, *J* = 11.2 Hz), 5.29 (1H, d, *J* = 11.6 Hz), 4.35 – 4.31 (2H, m), 4.19 (1H, d, *J* = 15.2 Hz), 3.73 (5H, br), 3.48 – 3.46 (1H, m), 3.26 – 3.20 (2H, m), 3.13 – 3.05 (4H, m), 2.72 – 2.50 (5H, m). FAB-HRMS *m/e* calcd for $[M + 2Cl^-]^+$ 1086.1833, found 1086.1823.

Preparation of the D4-tag model peptides

D4-tag peptide (Boc-DDDD-NH_2): The Boc-DDDD-NH_2 was synthesized by standard Boc chemistry in solution phase with Boc-Asp(Obzl)-OH and H-ASP(Obzl)-$CONH_2$ as the starting materials. Detailed synthetic procedure and compound characterization was reported previously.[15]

$(D4)_2$-tag peptide (Ac-DDDD-G-DDDD-GY-NH_2): The $(D4)_2$ peptide (Ac-DDDD-G-DDDD-GY-NH_2) was synthesized by an automated peptide synthesizer (ABI 433A, Applied Biosystems) using the standard Fmoc-based FastMoc coupling chemistry (0.1 mmol scale). The coupling reactions were performed with Fmoc Amide Resin (Applied Biosystems) using 4 equiv of amino acid and 4 equiv of 1-hydroxybenzotriazole (HOBt). After acetylation by the treatment with Ac_2O in CH_2Cl_2, the peptide cleavage and side-chain deprotection were carried out by treatment with 0.5 mL of TFA containing *m*-cresol (0.13 mL) and thioanisole (0.38 mL), and ethanedithiol (0.75 mL) over 1 h at room temperature. Crude peptide was precipitated in *tert*-butyl methyl ether and purified by reversed-phase HPLC (column; YMC-pack ODS-A, 250 x 20 mm, mobile phase; CH_3CN (containing 0.1% TFA) / H_2O (containing 0.1% TFA) = 5:95 $\rightarrow$ 35:65 (linear gradient over 40 min), flow rate; 9.9 mL/min, detection; UV (220 nm). Molecular weight of the peptide was confirmed by MALDI-TOF mass spectroscopy: calcd (found) for $C_{70}H_{60}N_{12}O_{29}$ $[M + H]^+$, *m/e* 1257.37 (1253.85).

Preparation of the tag-fused RNases: The tagged RNases were prepared from an equivalent amount of S-protein with tag sequences appended S-peptides by a self-assembling manner.[S1] The S-peptides tethering a D4-tag (DDDD), $(D4)_2$-tag (DDDD-G-DDDD-G) and His_6-tag (HHHHHH) at their N-termini were synthesized by the automated peptide synthesizer and purified by reverse-phase HPLC. Detailed synthetic

procedures and compound characterizations were reported previously.[15]

Fluorescence titration of 1-2Zn^{II} with the tag-fused RNase

Fluorescent spectra were recorded on a Perkin-Elmer LS55 spectrometer. All titration experiments were carried out in a quart cell (total volume 3 mL) at 25 °C. Due to the low solubility of the tag-fused RNases, which prevents it from preparation of the concentrated stock solution, the fluorescent measurements were carried out by the serial dilution of an aqueous buffer solution (50 mM HEPES, pH 7.2) containing **1**-2Zn^{II} (2 mM) and tag-fused RNases (4 μM) with a solution of **1**-2Zn^{II} (2 μM) to keep the concentration of **1**-2Zn^{II} at 2 μM in a cell. Plot of the excimer emission intensity at 472 nm (l_{ex} = 345 nm) was analyzed by a curve-fitting analysis assuming 1:2 binding to afford the first (K_1, M^{-1}) and second (K_2, M^{-1}) binding constant (Figure S3).

Visual detection of the (D$_4$)2-tagged RNase by using pyrene excimer emission

The aqueous solutions (50 μL, 50 mM HEPES, pH 7.2) containing 15 μg/mL of each protein were spotted on a micro slide glass (spot size; 6 mm diameter, purchased from Matsunami Glass Company). The slide glass was placed on a UV lamp (l_{ex} = 365 nm, 30 W) and the photos were taken by a digital camera in the dark.

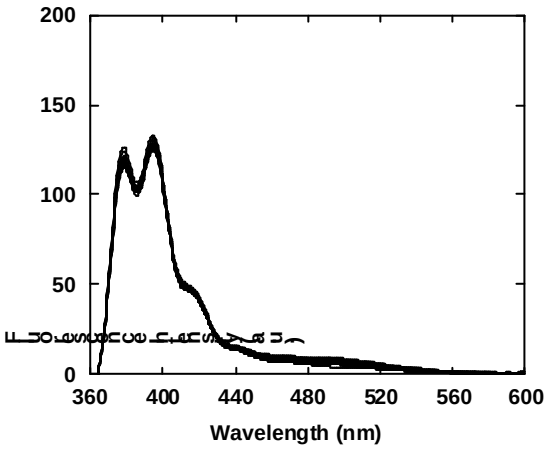


Figure S1. Fluorescence spectral change of **1**-2Zn^{II} (2 μM) upon addition of the D4 peptide (0 ~ 20 μM) in 50 mM HEPES buffer (pH 7.2), at 25 ^{o}C, l_{ex} = 345 nm.

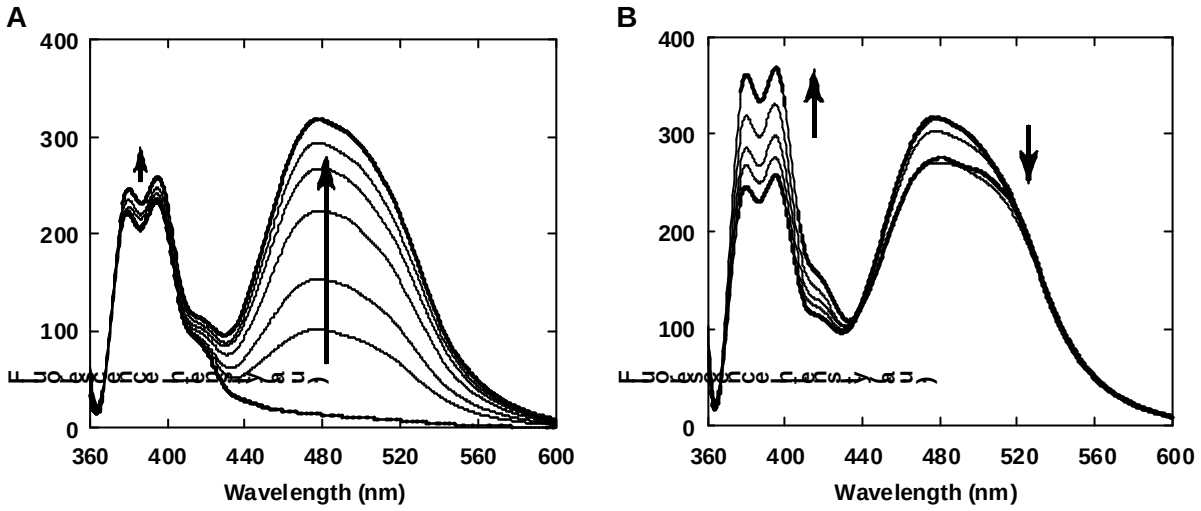


Figure S2. Fluorescence spectral changes of **1**-2Zn^{II} (2 μM) upon addition of the $(D4)_2$-RNase in 50 mM HEPES buffer (pH 7.2), at 25 ^{o}C, l_{ex} = 345 nm. A) [$(D4)_2$-RNase] = 0, 0.2, 0.4, 0.6, 0.8 and 1 μM. B) [$(D4)_2$-RNase] = 1, 1.5, 2, 3, 4 μM.

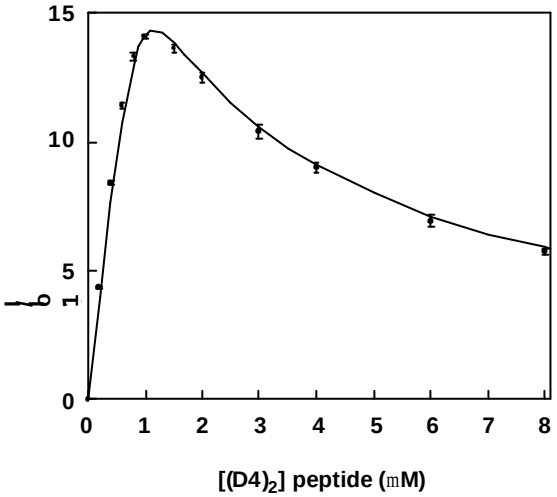


Figure S3. Curve fitting analysis of the fluorescence intensity change of **1**-2Zn^{II} (2 μM) upon addition of the $(D4)_2$ peptide in 50 mM HEPES buffer (pH 7.2).

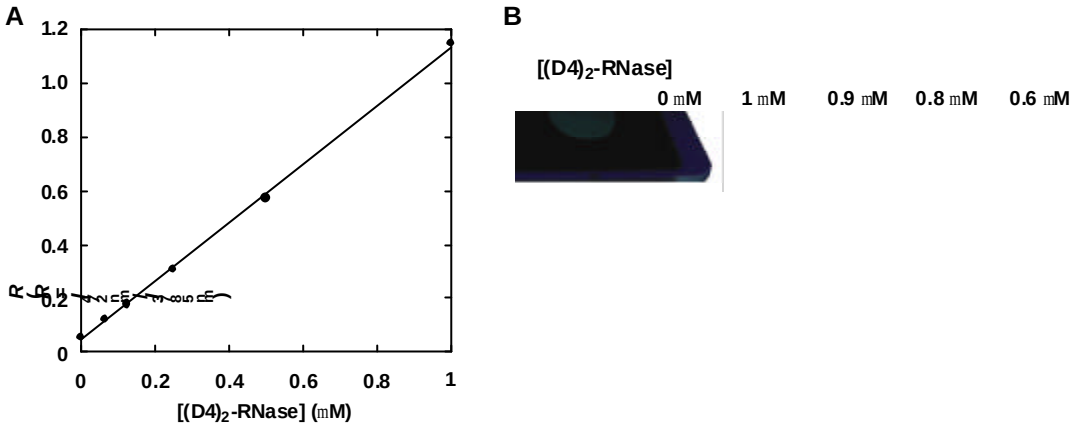


Figure S4. A) Plot of the emission ratio *R* ($I_{472\ nm}$ / $I_{378.5\ nm}$) of **1**-2Zn^{II} against the concentration of the $(D4)_2$-RNase. Conditions; 50 mM HEPES, pH 7.2, 25 °C, λ_{ex} = 345 nm. B) Visual detection of the $(D4)_2$-RNase using the binding-induced excimer emission of **1**-2Zn^{II} (10 μM). The concentration of the $(D4)_2$-RNase was changed from 0 to 1 μM.

References

[S1] a) P. R. Connelly, R. Varadarajan, J. M. Sturtevant, F. M. Richards, *Biochemistry* **1990**, 29, 6108-6114. b) I. Hamachi, R. Eboshi, J. Watanabe, S. Shinkai, *J. Am. Chem. Soc.* **2000**, 122, 4530-4531.