Electronic Supplementary Material (ESI) for Sensors & Diagnostics.

Paper-based Sensing of Pancreatic-cancer Biomarker α-Chymotrypsin through *Turn-on* Lanthanide-Luminescence

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Materials and methods

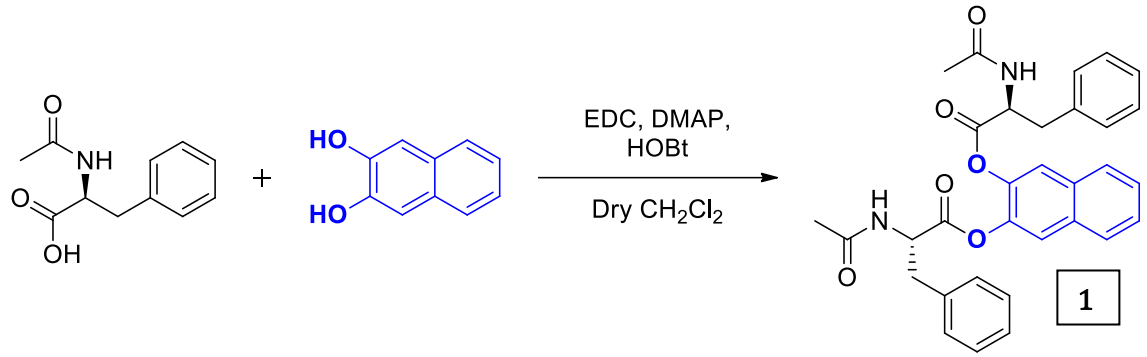
Terbium acetate (99.9% trace metals basis), sodium cholate (NaCh, from bovine and/or bovine bile, ≥99% purity), 2,3-dihydroxy naphthalene (DHN, recrystallized), 4-(Dimethylamino)pyridine (DMAP, purity ≥99.0% GC), α-chymotrypsin from bovine pancreas (Type II, lyophilized powder, ≥40 units/mg protein), β-glucosidase (6 U/mg, from almonds, mol wt ~135 000), lipase (2.9 U/mg, from Candida rugosa, mol wt ~67 000, alkaline phosphatase from bovine intestinal mucosa (lyophilized powder, ≥10 DEA units/mg solid), Urease (Type III, powder, 15,000-50,000 units/g solid from Canavalia ensiformis, Jack bean) were purchased from Sigma Aldrich. Whatman 3 paper was purchased from GE Healthcare.

Absorption spectra were recorded on a UV-3600 Shimadzu UV-Vis NIR spectrometer using 1 cm path length quartz cuvettes. Time-delayed emission for the gel samples was recorded on a Varian Cary Eclipse spectrometer in phosphorescence mode (delay time 0.2 ms, gate time 5.0 ms) and Varioskan® Flash Spectral Scanning Multimode Reader in time-resolved fluorescence (TRF) mode (delay time 0.2 ms, integration time 1.0 ms, bandwidth 12 nm). Emission from the gel-coated paper discs was recorded in 96 well plate on Varioskan® Flash Spectral Scanning Multimode Reader in TRF mode (delay time 0.2 ms, integration time 1.0 ms, bandwidth 5 nm). HPLC analysis was performed using a Shimadzu HPLC System (SCL-10A VP), consisting of a binary pump and a diode-array detector (DAD) and equipped with a Gemini C18 column (5 μm, 250 × 4.6 mm). ^{1}H NMR and ^{13}C NMR spectra were recorded at 400 MHz and 100 MHz, respectively. Milli Q water was used for all the measurements.

Synthesis of pro-sensitizer 1

N-Acetyl-L-phenylalanine was prepared according to the reported method[1] as white solid. A *bis*-phenylalanine ester derivative of DHN was prepared by Steglich esterification of N-Acetyl-L-phenylalanine with 2,3-dihydroxynaphthalene. To achieve this, N-Acetyl-L-phenylalanine

(200 mg, 0.965 mmol) was dissolved in dichloromethane (10 mL) under an Argon atmosphere. 1-Ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (185 mg, 0.965 mmol) and 1-



Scheme S1: Synthetic scheme for *pro*-sensitizer **1**.

hydroxybenzotriazole (130 mg, 0.965 mmol) were added, followed by the addition of DMAP (11 mg, 0.0965 mmol). The reaction mixture was stirred at 0 °C for 40 min. DHN (77 mg, 0.48 mmol) was added and stirred at 0 °C for 30 min. After the removal of the solvent under vacuum, the reaction mixture was diluted with diethyl ether (50 mL x 2), after which the ether layer was washed with 2M HCl (50 mL x 2) and water (50 mL x 2) and then dried over anhydrous Na_2SO_4. The solvent was evaporated after filtration, and the crude product was purified by column chromatography on silica gel using 30 % ethyl acetate-hexane mixture to yield compound **1** (80 mg, 31%) as a white solid. As the corresponding mono-ester derivative itself turned out to be a promising sensitizer for Tb (III), the di-ester derivative (**1**) was chosen as the preferred "*pro*-sensitizer," which did not show any appreciable sensitization.

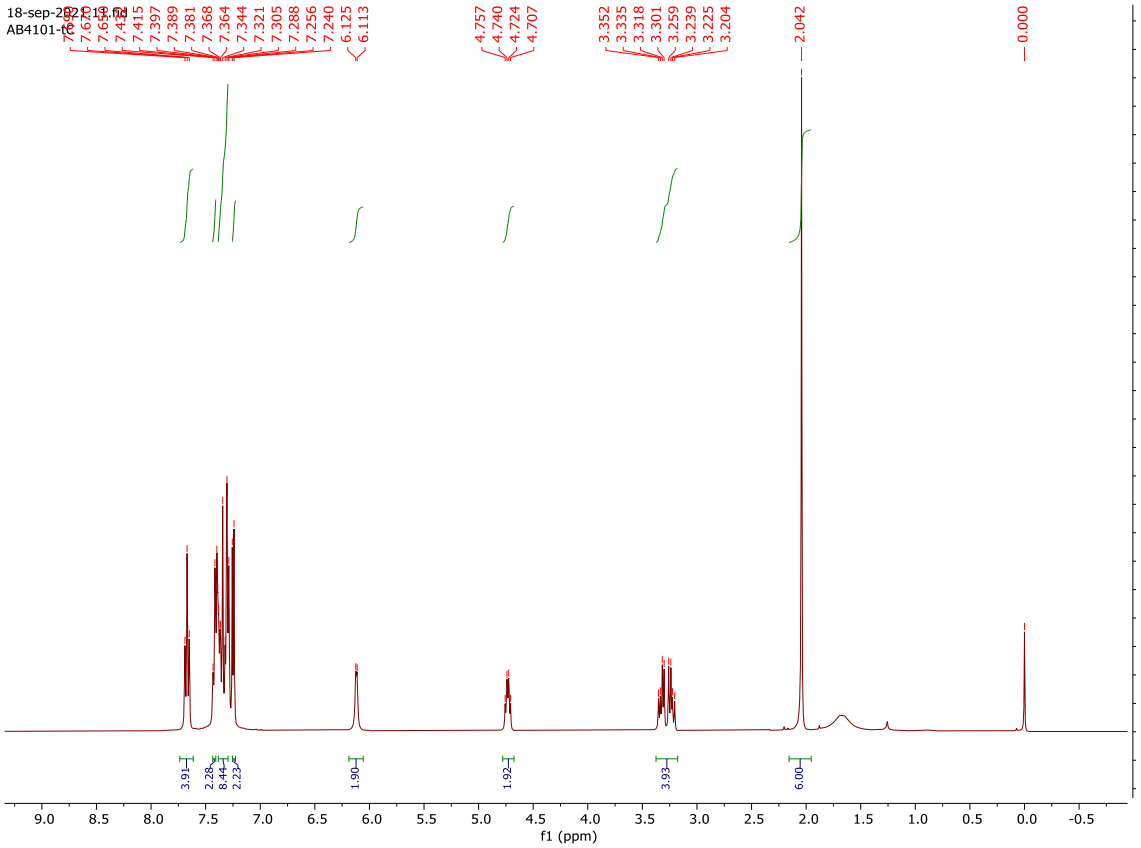
^{1}H NMR (400 MHz, $CDCl_3$) δ: 7.67 (t, J=8 Hz, 4H), 7.43-7.29 (m, 10H), 7.24 (s, 2H), 6.11 (d, J=4.8 Hz, 2H), 4.72 (q, 2H), 3.35-3.20 (m, 4H), 2.04 (s, 6H).

^{13}C NMR (100 MHz, $CDCl_3$) δ: 170.62, 169.48, 140.37, 135.95, 131.47, 129.30, 128.83, 127.52, 127.34, 126.57, 120.73, 53.60, 37.53, 29.70, 22.96.

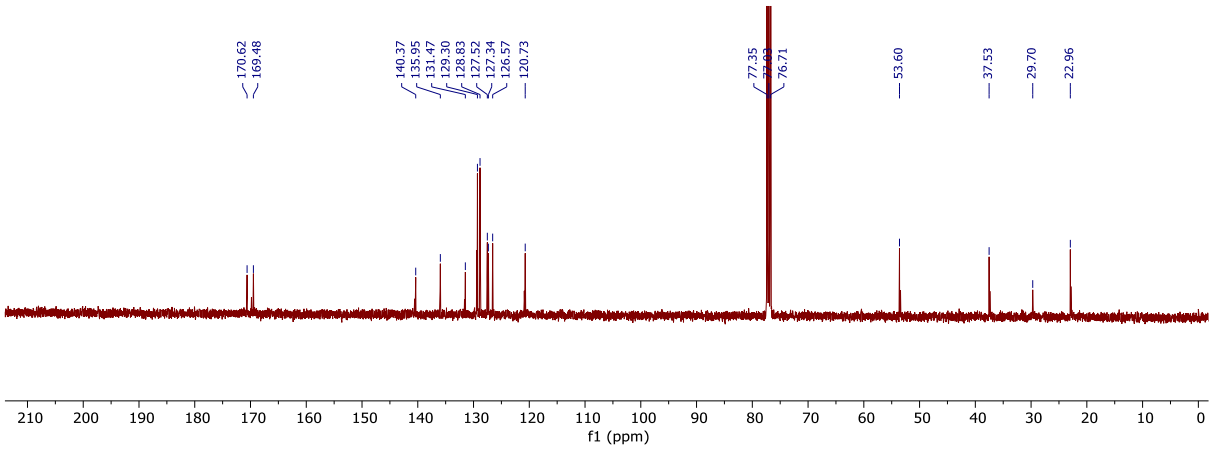
IR (KBr, cm^{-1}): 1721, 1652, 1159, 696

HRMS: Calculated for $C_{32}H_{30}O_6N_2K$ [M+K] 577.1741; observed $C_{32}H_{30}O_6N_2K$ [M+K] 577.1742.

^{1}H NMR spectrum of the *pro*-sensitizer 1: (400 MHz, $CDCl_3$)



^{13}C NMR spectrum: (100 MHz, $CDCl_3$)



Procedure for gel-based assay

Method 1: Fresh stock solutions of *pro*-sensitizer **1** and α-Chymotrypsin were prepared in 30 mM Na-Ch solution. These solutions (100 µL each) were mixed in a test tube (7.5 cm × 1.2 cm) to which 200 µL of 10 mM $Tb(OAc)_3$ was added. Then, the enzyme and *pro*-sensitizer were incubated at 25 °C for 30 min in an oil bath in the as-prepared Tb-Ch gel. The final concentrations of the components and analytes in the gels were: 5 mM Tb (III), 15 mM cholate, *pro*-sensitizer **1** 37.5 µM.

Method 2: For enzyme assay in Na-Ch solution, *pro*-sensitizer **1** and α-Chymotrypsin in 30 mM Na-Ch (100 µL each) were incubated at 25 °C for 30 min in an oil bath. After the incubation, 200 µL of 10 mM $Tb(OAc)_3$ was added.

Procedure for kinetics study

Fresh stock solutions of *pro*-sensitizer **1** and α-chymotrypsin solution were prepared in 30 mM NaCh. These solutions (100 µL each) were mixed in a test tube (7.5 cm × 1.2 cm), followed by the addition of 200 µL of 10 mM Tb $(OAc)_3$ solution. Then, the incubation was performed in gel matrix at 25 °C for 1-5 min in an oil bath. The TbCh gel (100 µL portions) were added into the wells of a 96 well plate, after which TRF measurements were made.

Paper-based sensing procedure

TbCh hydrogel (5/15 mM) doped with the *pro*-sensitizer **1** was prepared by mixing 200 µL of 75 µM of *pro*-sensitizer 1 in 30 mM Na-Ch solution with 200 µL of Tb $(OAc)_3$ (10 mM) in a test tube (7.5 cm × 1.2 cm) and sonicating in a bath sonicator (1.5 L) at 5-10 °C for 5-6 s. After stabilizing the gel at RT for 10 min, the gel was sonicated again (5-6 s), to reduce its viscosity. 20 µL of this weak gel was drawn using a micropipette and drop casted on 3.5 mm diameter discs (discs were cut from Whatman 3 paper using a standard one-hole office punch). The paper absorbed the gel in 30 min and was transferred to a 96 well plate. α-chymotrypsin solution (10 µL, prepared in NaCh) was added on each disc, and emission measurements were recorded using a plate reader after 15 min of air drying.

Molecular docking

Enzyme structure (PDB ID: 1YPH) was obtained from the Protein Data Bank, and molecule 1 was constructed and optimized by ChemDraw 3D and then converted to PDB format. The widely accepted molecular grid-based docking program Autodock4.2 was used to predict the binding modes and binding free energies for the screened enzyme α-Chymotrypsin. Ligand PDB formats were edited in AutoDock Tools by setting the root, torsion, and Gasteiger charges and finally saved as PDBQT formats. For the enzyme, water molecules were removed, polar hydrogen was added, and Kollman charges were calculated with AutoDock Tools. The grid size was set to be 30× 32× 36 for all the simulated docking studies. During docking, the number of Genetic Algorithms runs to get the maximum number of generations for the ligand-protein complex. The ranking of poses was done based on binding energies, cluster analysis, and the non-covalent interactions between the ligand and the receptor. The interactions were visualized using MGL Tools-1.5.6, PyMOL and Discovery Studio 2019 Client, and LigPlot. The binding model analysis for the pro-sensitizer was obtained from the best poses selected.

Absorption spectra of pro-sensitizer 1 and DHN

The absorption spectra of the pro-sensitizer **1** and sensitizer DHN in 30 mM Sodium cholate solution were recorded. These showed a similar spectral pattern but a significant increase in absorbance value after the diester synthesis.

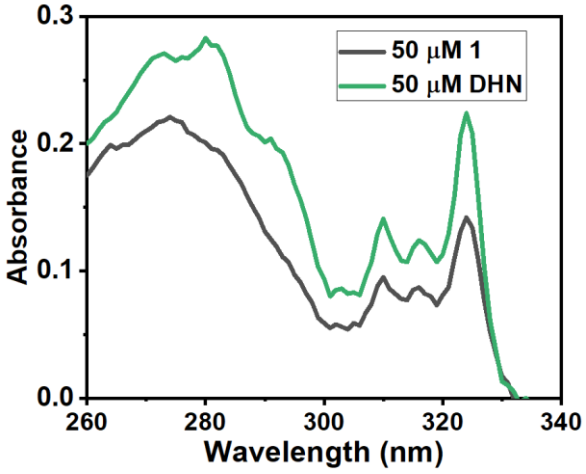


Figure S1: Absorption spectra of 50 μM of DHN and compound **1** in 30 mM Sodium cholate

Stability of *pro*-sensitizer 1 when incubated in Tb-Ch at 25 °C for 60 min:

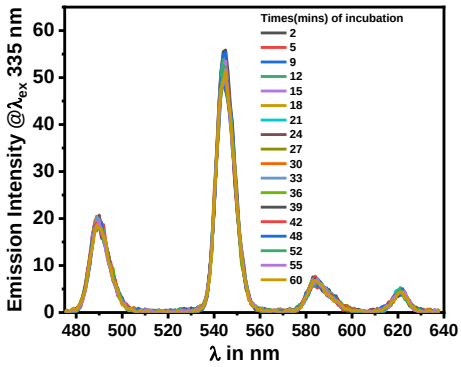


Figure S2: Emission spectra for Tb^{3+} -luminescence (@ λ_{ex} 335 nm) of 37.5 µM *pro*-sensitizer doped Tb-Ch gel.

Optimization of α-chymotrypsin assay

Method 1:

Incubation in gel medium

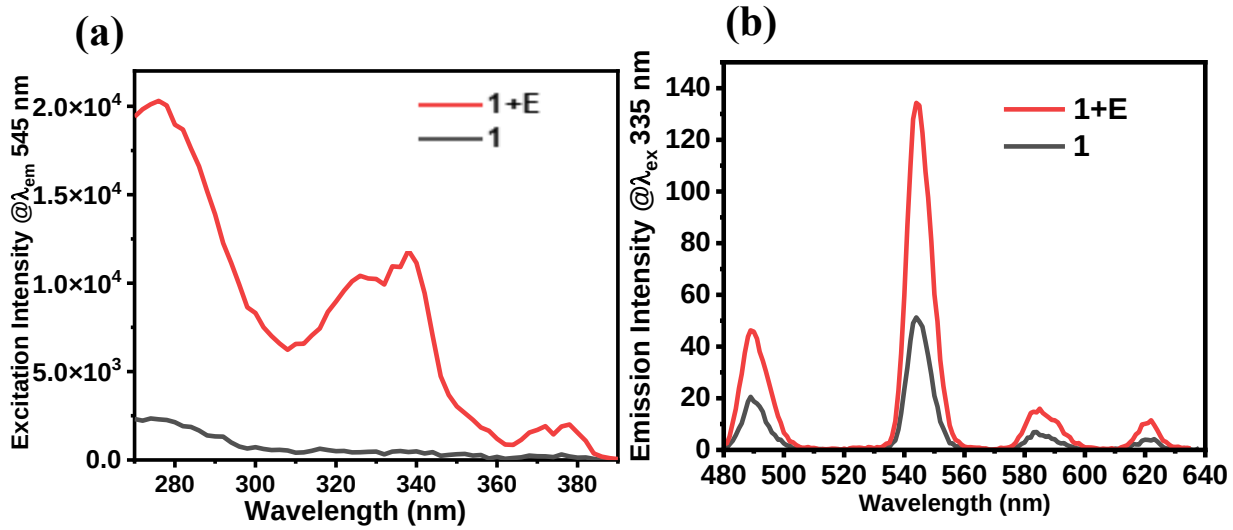


Figure S3: a) Excitation Spectra and b) Emission spectra (λ_{ex} 335 nm) of TbCh (5/15 mM) gel after incubation of **1** with α-chymotrypsin (E), and water. Final incubation concentrations of **1** and **α-chymotrypsin** were 37.5 µM and 5 µg/mL, respectively.

Time-dependent assay in method 1

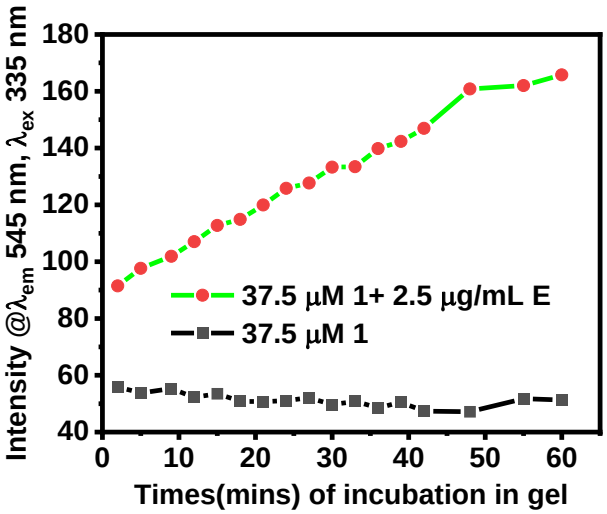


Figure S4: Time dependent emission Intensity (λ_{ex} 335 nm, λ_{em} 545 nm) of TbCh (5/15 mM) gel after incubation of **1** with α-chymotrypsin (E), and water. Final incubation concentrations of **1** and **α-chymotrypsin** were 37.5 μM and 5 μg/mL, respectively.

Method 2:

Incubation in 30 mM Na-Ch

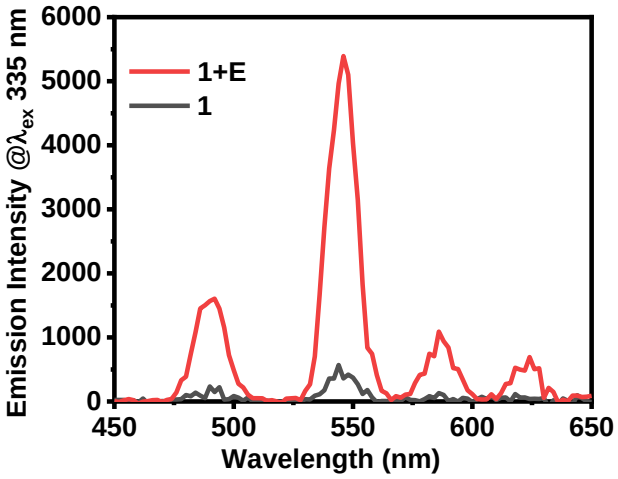


Figure S5: Emission spectra (λ_{ex} 335 nm) of TbCh (5/15 mM) gel after incubation of **1** with α-chymotrypsin, and water. Final incubation concentrations of **1** and **α-chymotrypsin (E)** were 37.5 μM and 5 μg/mL, respectively.

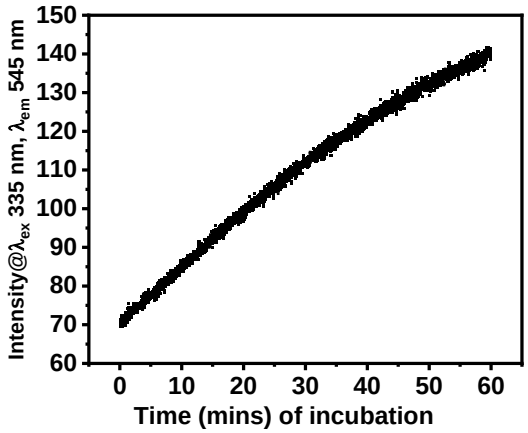


Figure S6: Kinetic scan of TbCh (5/15 mM) gel after incubation of **1** with α-chymotrypsin. Final incubation concentrations of **1** and **α-Chymotrypsin** were 37.5 µM and 5 µg/mL, respectively.

Time dependent assay in Method 2

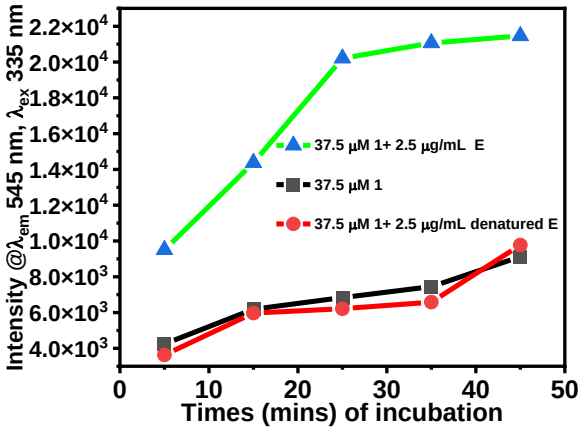


Figure S7: Time dependent emission Intensity (λ_{ex} 335 nm, λ_{em} 545 nm) of TbCh (5/15 mM) gel after incubation of **1** with α-chymotrypsin, and water and denatured enzyme. Final incubation concentrations of **1** and **α-chymotrypsin** were 37.5 µM and 5 µg/mL, respectively.

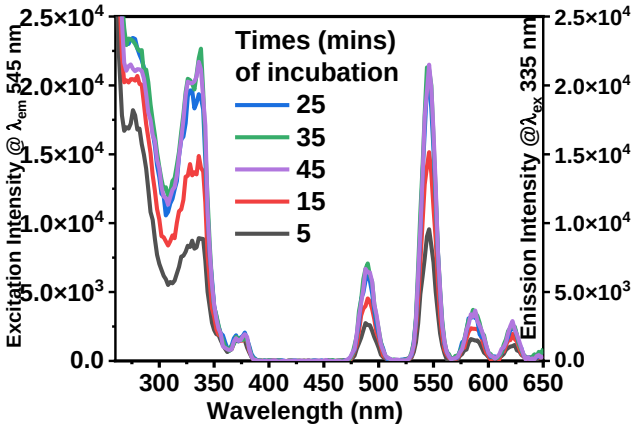


Figure S8: Luminescence spectra of TbCh (5/15 mM) gel after incubation of **1** with α-chymotrypsin, and water. Final incubation concentrations of **1** and **α-Chymotrypsin** were 37.5 µM and 5 µg/mL, respectively.

Kinetic Study

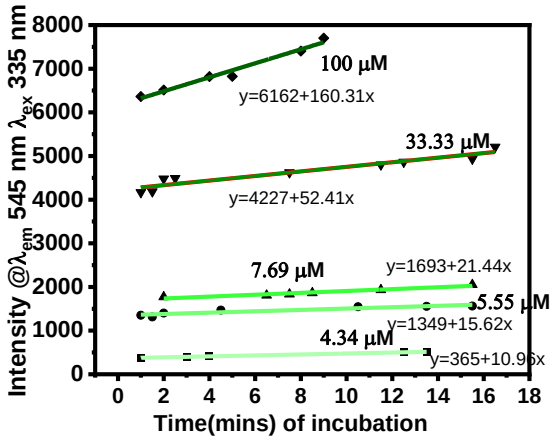


Figure S9: α-Chymotrypsin (10 µg/mL) assay with different concentrations of **1** in Tb-Ch gel system (5 mM:15 mM) at 298 K.

Table S1: Literature data of **K_M values** (with different substrates)

Assay Method	**K_M value (µM)**	**Chymotrypsin source**	**Reference**
Fluorescence	59	*Bos taurus*	2
Fluorescence	87	Not mentioned	3
Fluorescence	16	Not mentioned	4
Tb(III)-luminescence	**85**	**bovine pancreas**	**This work**

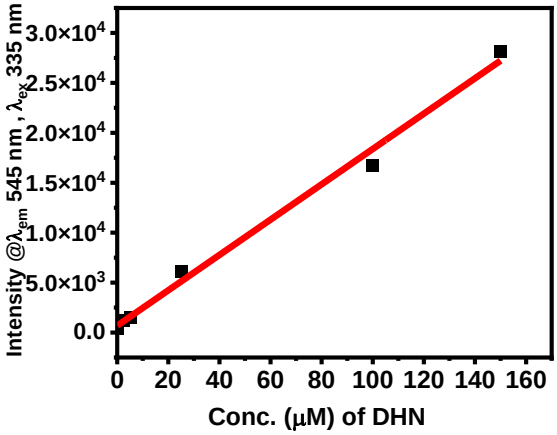


Figure S10: Calibration plot for Tb (III) luminescence at 545 nm (λ_{ex} 335 nm) with increasing DHN concentration in 5/15 mM TbCh gel.

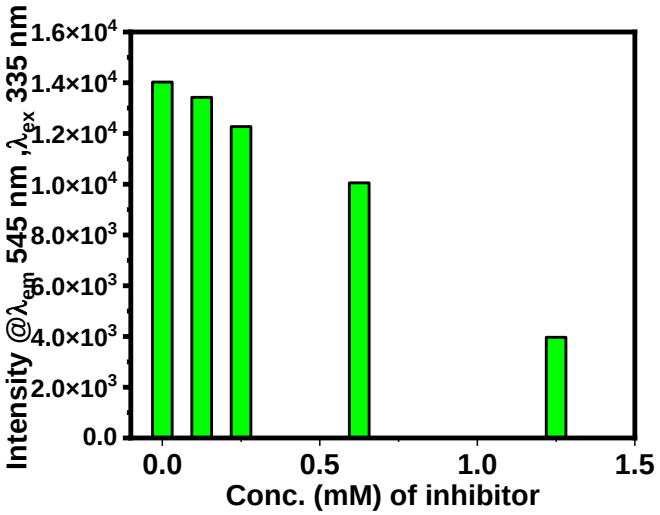


Figure S11: (a) Emission Intensity at 545 nm (λ_{ex} 335 nm) for α-chymotrypsin assay in the presence of varying concentrations of **Inhibitor.** Final incubation concentration of **1** and α-chymotrypsin were 37.5 µM and 25 µg/mL, respectively.

Calculation formula:

Inhibition efficiency= $[100 - \{(F_I - F_0/F_E - F_0) * 100\}]$

where,

F_I is the fluorescence intensity in the presence of Inhibitor and α-chymotrypsin.

F_0 is the initial fluorescence intensity without Inhibitor and α-chymotrypsin.

F_E is the fluorescence intensity in the presence of α-chymotrypsin without Inhibitor.

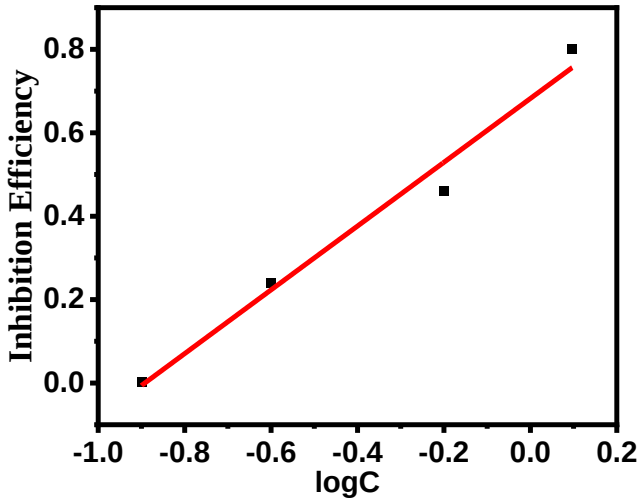


Figure S12: A plot of inhibition ratio versus the logarithm of inhibitor

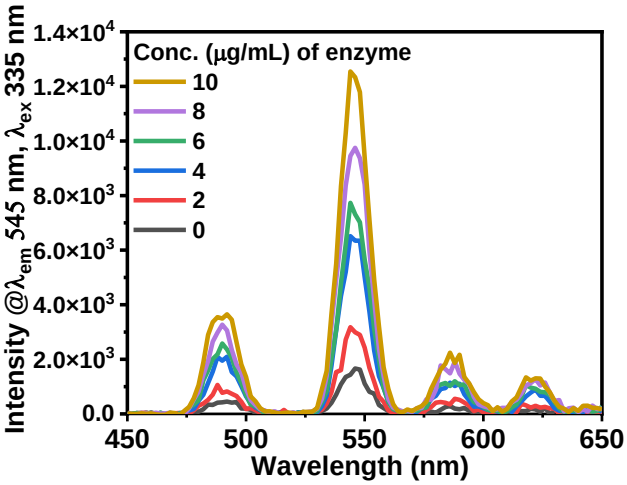


Figure S13: Emission spectra for Tb^{3+} -luminescence @ λ_{ex} 335 by incubating 37.5 μM probe with various concentrations of enzyme.

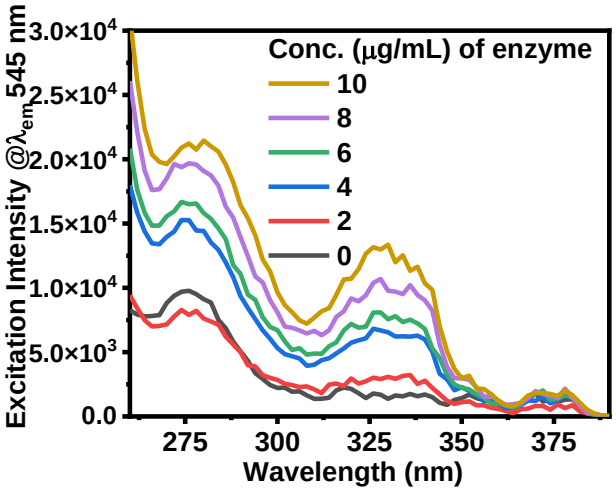


Figure S14: Excitation spectra for Tb^{3+} -luminescence @ λ_{em} 545 nm by incubating 37.5 μM probe with various concentrations of enzyme.

Table S2: Comparison of LOD with other reported non-peptide-based probes

Detection Method		**LOD value**	**Reference**
Fluorescence		50 ng/mL	2
Fluorescence		0.013 U/mL or 13.5 ng/mL	3
Fluorescence		67 ng/mL	4
Fluorescence		8.4 ng/mL	5
Time gated Tb (III)-luminescence	Gel based matrix	370 ng/mL or 0.014 U/ mL	This work
	Paper based sensor	330 ng/mL or 0.012 U/ mL	This work

HPLC Studies

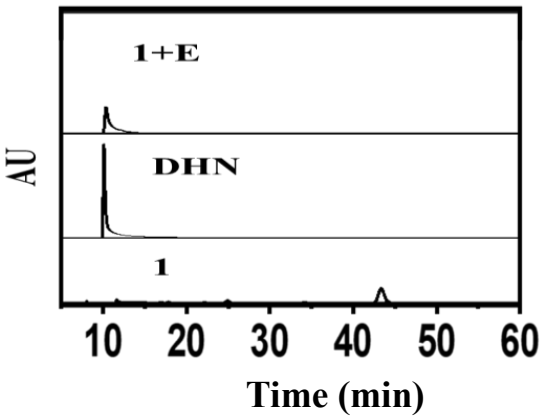


Figure S15: HPLC chromatogram (monitored at 270 nm).

1: 1.8 mM *pro*-sensitizer **1**, DHN: 1.8 mM 2,3-dihydroxynaphthalene, **1**+E: 150 µM **1** incubated with E(α-chymotrypsin) 20 µg/mL, mobile phase: 50 % MeOH/H_2O

3.3.12 Molecular Docking

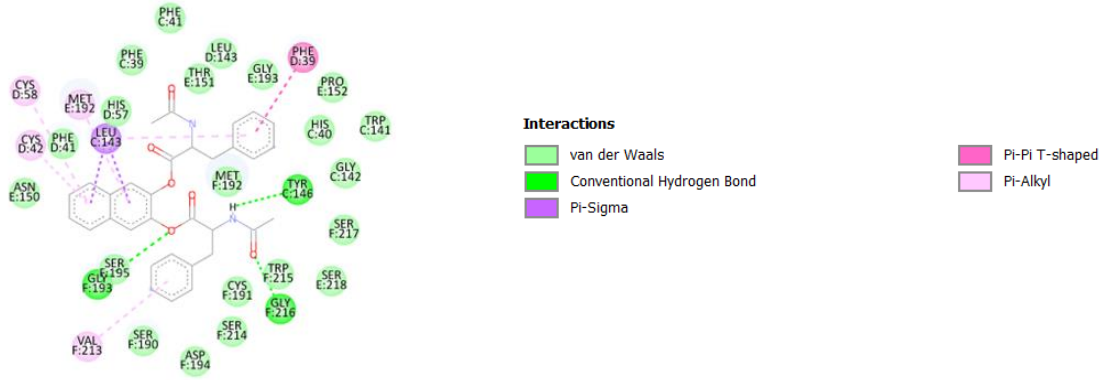


Figure S16: Simulated binding models of probes **1** in the active site of α-Chymotrypsin.

Material cost for coated disc preparation

Material	Cost for a Single Disc (INR)
Whatman paper grade 3 (38 mm^2)	0.03
Sodium cholate (0.267 mg)	0.017
Tb (III) acetate (0.067 mg)	0.140
Pro-sensitizer **1** + other reagents + X	0.002
Total	0.189

Material cost was calculated based on Aldrich prices.

X include cost of reagents, starting materials, silica gel and solvent used for column purification. The time spent by this author and instrument operators were converted into cost based on their current salaries.

References:

(1) K. C. Cartwright, J. A. Tunge, *ACS Catal.* 2018, **8**, 11801–11806.

(2) L. Wu, S. H. Yang, H. Xiong, J. Q. Yang, J. Guo, W. C. Yang, G. F. Yang, *Anal. Chem.* 2017, **89**, 3687–3693.

(3) S. Mu, Y. Xu, Y. Zhang, X. Guo, J. Li, Y. Wang, X. Liu, H. Zhang, *J. Mater. Chem. B* 2019, **7**, 2974–2980.

(4) S. Y. Liu, X. Zou, X. Gao, Y. Y. Zhan, *Anal. Chem.* 2022, **94**, 17922−17929.

(5) Y. Chen, J. Cao, X. Jiang, Z. Pan, N. Fu, *Sensors Actuators, B Chem.* 2018, **273**, 204–210.