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Electronic Supplementary Information

Dipolar Coupling-Based Electron Paramagnetic Resonance Method for Protease Enzymatic Characterization and Inhibitor Screening

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Experimental Procedures

Materials and reagents

Unless otherwise stated, all reagents and solvents used for synthesis were purchased from commercial sources and used without further purification. Proteases including PAC-1、2-HBA、Belnacasan、Z-IDTE-FMK、Z-VAD(Ome)-FMK、Q-VD-Oph、Z-DEVD-FMK were purchased from MedChemExpress. MTSL (1-oxyl-2,2,5,5-tetramethyl-Δ3-pyrr-oline-3-methyl methanethiosulfonate) was purchased from Toronto Research Chemicals, Ontario, Canada.

Synthesis of DOTA-DEVD-C

Peptide DOTA-DEVD-C was synthesized by KS-V Peptide Biological Technology Co., Ltd (Hefei, China) by standard solid phase peptide synthesis (SPPS) procedure. 2-CTC resin was swelled in DMF/DCM 1:1 for 30 min. Fmoc-Cys(Trt)-OH (4eq, according to the substitution rate of the resin) and DIEA (8eq) dissolved in DMF and reacted with the resin overnight to load Cys residue onto the resin. Fmoc de-protection was carried out using 20 v%/v% piperidine in DMF twice (5min+10min). Fmoc-Asp(OtBu)-OH, Fmoc-Val-OH, Fmoc-Glu(OtBu)-OH, Fmoc-Asp(OtBu)-OH were coupled onto the resin followed by Fmoc de-protection successively, coupling condition was Fmoc-AA (4 eq), HCTU (3.8 eq) and DIEA (8 eq) in DMF for 45min. DOTA-tris(tBu)ester was reacted with the same coupling condition.

The resin was washed thoroughly by DCM and dried in vacuum. TFA cleavage cocktail was poured into the reaction vessel and react for 3h. TFA cocktail was filtered iInto a 50 mL EP tube and TFA was removed by blowing N_2 gas. Cold ether was poured into the tube to precipitate the crude peptide. The Crude peptide was purified by reversed-phase HPLC (eluent: H2O/acetonitrile containing 0.1% TFA) and characterized by ESI-MS.

Synthesis of Gd-DOTA-DEVD-C

43mg purified peptide DOTA-DEVDC was dissolved in 22.5 mL 0.1M HEPES buffer, and 18.37 mg GdCl3.H2O was added. The reaction solution was stirred at room temperature for 4 hours. The reaction was purified by HPLC and the correct product was collected and lyophilized to obtain the product as white powder. ESI-MS 1121.10/1143.10(+Na^+).

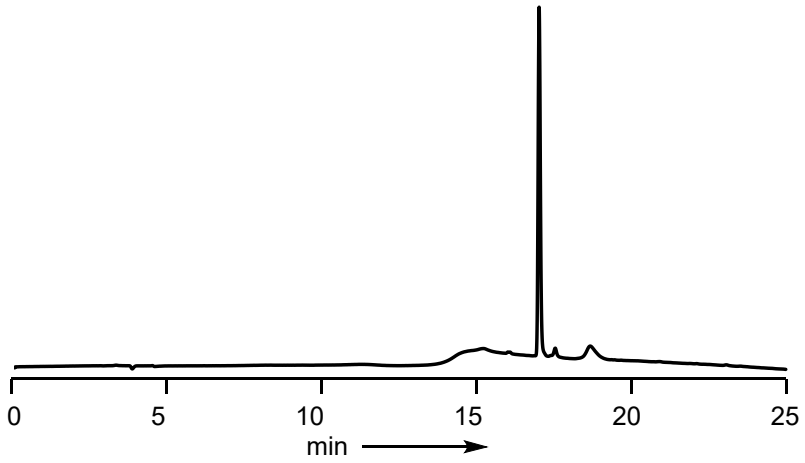


Figure S1. HPLC of peptide Gd-DOTA-DEVD-C
Eluent: 0.1% TFA aq. (0 min) → 99% acetonitrile / 1 % water containing 0.1% TFA (30min).

Generation of Gd-DOTA-DEVD-CysMTSL

Nitroxide MTSL was introduced into the thiol groups of purified Gd-DOTA-DEVD-C with a spin label/peptide molar ratio of 10:1 at 4 ℃, overnight. Excess spin label was removed using PD-10 gravity flow desalting columns (GE Biosciences). The

successful labeling of the probe was confirmed by analytical reverse-phase HPLC (a linear gradient from 10%-69% acetonitrile with 0.1% TFA, 30 min) and Mass spectroscopy. The main peak was detected by ESI-MS, calc. for 1379.18, found 1377.90.

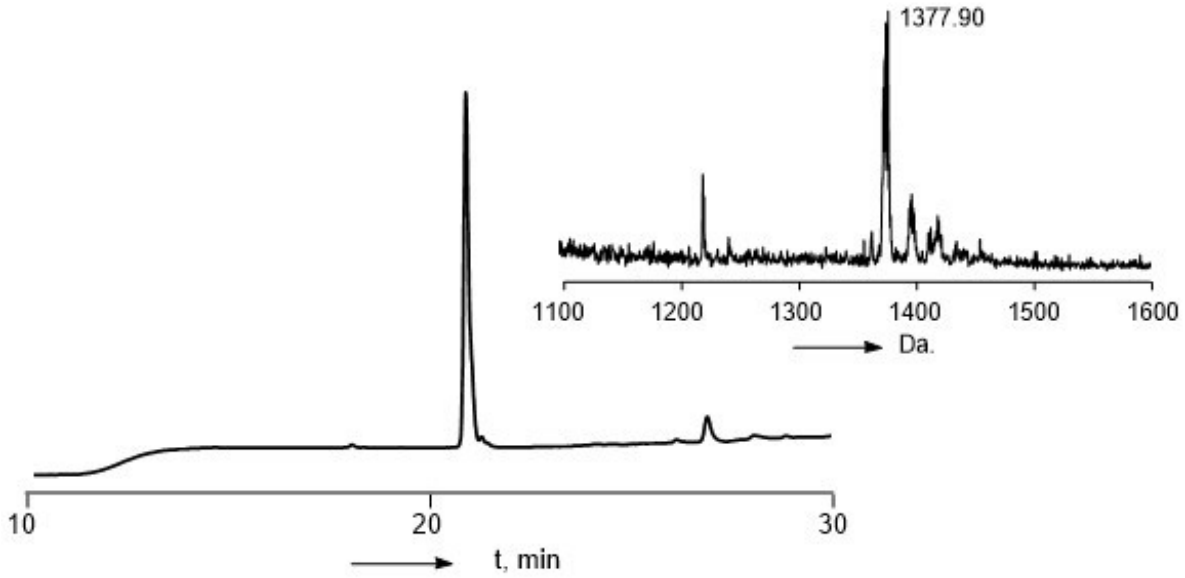


Figure S2. HPLC of probe Gd-DOTA-DEVD-CysMTSL and Mass spectroscopy.
Eluent: 0.1% TFA aq. (0 min) 10%-69% acetonitrile).

Synthesis of the cleavage-resistant probe Gd-DOTA-DEVA-CysMTSL

A cleavage-resistant probe was also designed and synthesized as the negative control. The probe was designated as Gd-DOTA-DEVA-CysMTSL, in which the last aspartic acid residue in DEVD sequence required for caspase-3 cleavage was mutated to alanine. In contrast to DEVD sequence, the DEVA sequence is not cleavable to caspase-3 so this probe is supposed to be “cleavage-resistant“.

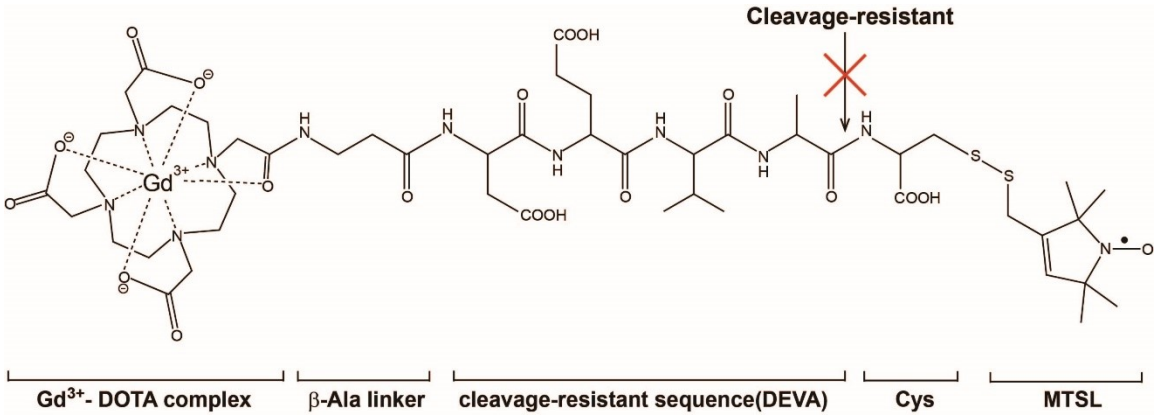


Figure S3. Structure of the Gd-DOTA-DEVAD-CysMTSL probe. This probe bears a DEVA sequence which is not specific for caspase-3 and therefore is cleavage-resistant to caspase-3.

The synthesis and labeling procedure is identical to that of Gd-DOTA-DEVD-CysMTSL except that the last aspartic acid residue in DEVD sequence required for caspase-3 cleavage was mutated to alanine during synthesis. The successful labeling of this cleavage-resistant probe was confirmed by analytical reverse-phase HPLC (a linear gradient from 10%-69% acetonitrile with 0.1% TFA, 30 min) and Mass spectroscopy. The main peak was detected by ESI-MS, calc. for 1335.18, found 1334.09.

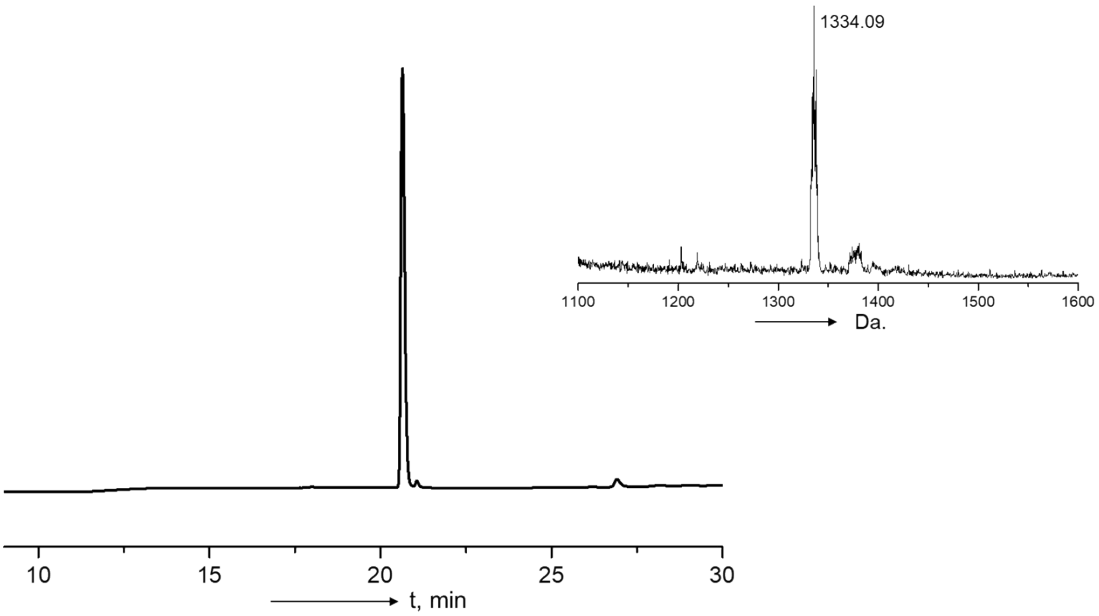


Figure S4. HPLC and Mass spectroscopy of cleavage-resistant probe Gd-DOTA-DEVA-CysMTSL. Eluent: 0.1% TFA aq. (0 min) 10%-69% acetonitrile).

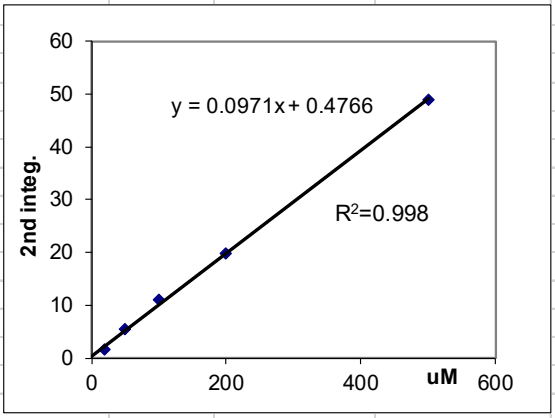
Labeling efficiency calculation

Briefly, the spin labeling efficiency was calculated by comparison of the area of the EPR absorption spectra of the labeled protein and a reference spin probe (TEMPO) of known concentration. The y-axis of the second integral was proportional to the number of spins in the measured probe[1]. Here, a Gd-depleted probe was labeled and used for labelling efficiency calculation. The number of spins was calculated by double integration of the resonance signal lines after background subtraction. The area under the absorption signal was converted to a radical concentration through a linear standard curve (R^2 = 0.998) based on solutions of 20–500 μM of the stable nitroxyl radical TEMPO (g-value of TEMPO 2.0060) (Sigma-Aldrich, USA). The labeling efficiency was analyzed by measuring the peptide concentration and the spin concentration, and the efficiency was approximately 97%.

EPR labeling ratio calculation

TEMPO standard

uM	2nd integ.
20	1.633
50	5.470
100	11.07
200	19.79
500	48.94



Linear Regression

m	b
0.097149	0.476649287

Samples	2nd integ.	Dilution	Spin CONC(uM)	Protein CONC(uM)	Labelling Efficiency
MTSL-labeled Probe	31.62	1	320.6	330	0.971432048

Figure S5. Calculation of labelling efficiency using TEMPO as standard.

Expression and purification of caspase-3

Caspase-3 was expressed and purified according to publised protocols. [2.3] Briefly, oligonucleotide encoding full-length human caspase-3 protein were synthesized by GENEWIZ (Suzhou, China) and were introduced into the plasmid expression vector pET28a (Novagen) and was verified by DNA sequencing. The plasmids were transformed into BL21 (DE3). Single colonies were used to inoculate a 5 ml 2 YT (0.5 g NaCl, 1% yeast extract, and 1.6% tryptone) pre-culture, containing the appropriate antibiotic and was then grown at 37 ℃ overnight (225 rpm). This culture was used to inoculate 500 ml of selective 2 YT medium in 2-liter baffled flasks and grown at 37℃. When the culture reached an absorbance of 0.6–0.8 the temperature was lowered to 30 C and expression was induced by the addition of 0.2 mM IPTG. Cells were harvested after 3 hours of incubation and then washed in a buffer containing 100 mM Tris–HCl, pH 8 and 100 mM NaCl and subsequently resuspended in 50 ml lysis buffer (100 mM Tris–HCl, pH 8, 100 mM NaCl, 20 mM imidazole, and 10 units/ml DNase I). Cells were broken using probe sonication and purified using Ni-NTA affinity chromatography (Qia-gen, USA). Nonspecifically bound protein was washed out with 100–200 column volumes wash buffer. The protein was eluted in 0.5 ml fractions with 6 ml elution buffer (50 mM Tris–HCl, pH 8, and 150 mM NaCl, and 250 mM imidazole). Fractions containing pure, active caspase were pooled. The concentration of the purified protein was determined by OD_{280}.

EPR measurements

Continuous wave (CW) EPR spectroscopy was performed on a Bruker A300 spectrometer (Bruker Biospin GmbH, Rheinstetten, Germany) at X-band (9.5 GHz) equipped with a high-sensitivity cavity (ER 4119HS, Bruker Biospin GmbH, Rheinstetten, Germany). The temperature was kept at 37 °C using a low dewar insert and a Bruker variable temperature control unit. Spectra were recorded at a microwave power of 2 mW(confimed no signal saturation) over a scan width of 65 Gauss with a field modulation of 1 Gauss at a modulation frequency of 100 kHz through all measurements. Samples were placed in a glass capillary tube with a fixed volume of 20 μL.

For EPR monitoring of the time-course of the enzymatic cleavage reaction, the probe was mixed with caspase-3 in reaction buffer(final volume 20 μL) and was immediately placed in EPR resonator. The EPR spectrometer was then critically coupled and a two-dimensional EPR spectrum (X: Field Sweep; Y: incremental Sweep) was collected until the EPR line intensity did not increase anymore (which means all probes have been cleaved). In this way, the EPR spectrum was collected in an “in-situ” way and all parameters (including Q value) was kept constant. The peak-to-peak amplitude of the central peak of EPR spectra, referring to as ΔA_{pp}, was calculated with a python script and plotted against reaction time.

Estimation of Michaelis constants k_M and the maximum rate of reaction V_{max}

The Michaelis constants k_M and the maximum rate of reaction V_{max} were estimated by fitting with Eq. (1) and Eq. (2) [4]:

$$V_0 = \frac{V_{\max}[S]}{k_M + [S]} \qquad \text{Eq. (1)}$$

$$V_{\max} = K_{cat}[E_0] \qquad \text{Eq. (2)}$$

Where V_{max} means the maximal rate of the enzyme catalysis reaction at a given concentration of enzyme. k_M, the Michaelis constant, is the concentration of the substrate required to ½ V_{max} , has units of M. *[S]* is the concentration of substrate. V_0 is the initial rate of the reaction while $[E_0]$ means the concentration of the enzyme at the beginning of reaction. K_{cat} is the first-order rate constant for the enzyme-catalyzed reaction at saturating concentrations of substrate.

Here, we assumed all substrate can be cleavage by the end of the reaction and initial velocities V_0 were measured for each curve and plotted against the concentration of nitroxide.

Estimation of reaction rate in the presence of different effectors

The change of EPR signal of reaction systme in the presence of different effectors has been normalized to compare with each other. Reaction system contains equal amount of effector and substrate(400 μM) and 5 μM of caspase-3 in HEPES

reaction buffer at 37 ℃. Data points in the presence of different effectors and corresponding fitting results (linear fit) were shown with symbols and lines with different colors, as indicated in Fig. S6.

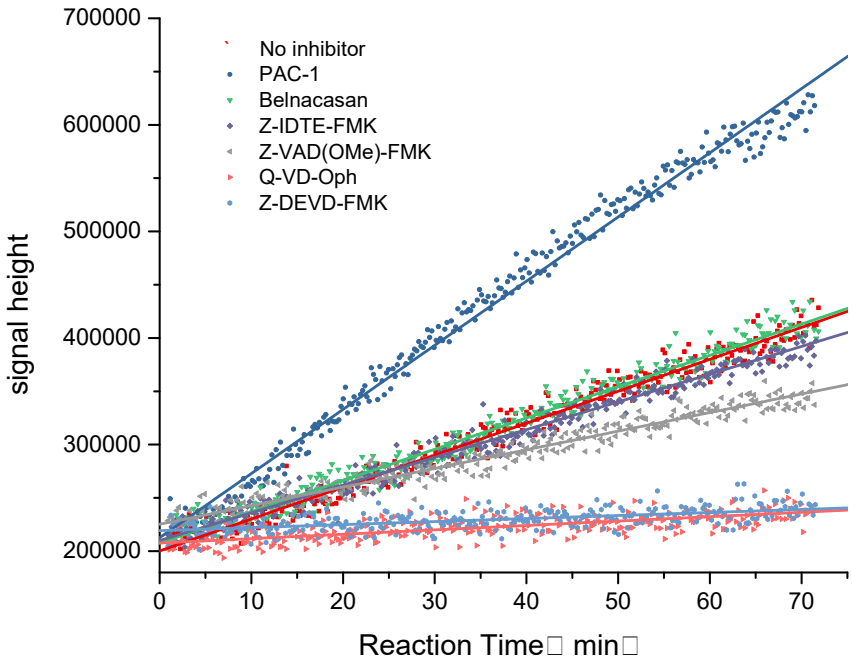


Figure S6. Change of EPR signal over a period of 70 minutes in the presence of different effectors.

References

[1] D. Yilmaz, A. I. Dimitrova, M. Walko, A. Kocer, *Eur Biophys J.*, 2015, **44**(7), 557-565.

[2] H. Roschitzki-Voser, T. Schroeder, E. D. Lenherr, F. Frolich, A. Schweizer, M. Donepudi, R. Ganesan, P. R. Mittl, A. Baici and M. G. Grutter, *Protein Expr Purif*, 2012, **84**, 236-246.

[3] D. Hwang, S. A. Kim, E. G. Yang, H. K. Song and H. S. Chung, *Protein Expression and Purification*, 2016, **126**, 104-108.

[4] J.R. Lorsch, Chapter One - Practical Steady-State Enzyme Kinetics, in: J. Lorsch (Ed.) Methods in Enzymology, Academic Press, 2014, pp. 3-15.