Supporting Information

Naphthalimide-derived Fluorogenic SNAP Probe for Real-time Monitoring Protein Degradation

Xuelian Zhou,[a,b] Lu Miao,[a,*] Yonghui Chen,[a] Jinjing Shi,[a,b] Qinglong Qiao,[a,*] and Zhaochao Xu[a,b,*]

[a] Dalian Institute of Chemical Physics, Chinese Academy of Sciences, 457 Zhongshan Road, Dalian 116023, China

[b] School of Chemistry, Dalian University of Technology, 2 Linggong Road, Dalian 116024, China

[*]Corresponding authors

E-mail address: miaolu@dicp.ac.cn; qqlqiao@dicp.ac.cn; zcxu@dicp.ac.cn

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Materials and methods

Materials and instruments.

Unless otherwise specifically stated, all reagents were purchased from commercial suppliers (Sigma-Aldrich, Aladdin, and Sangong Biotech) and used without further purification. All water used was from a Millipore water purification system with a minimum resistivity of 18.0 MΩ·cm. The plasmids pCMV-pSNAPf and pET22b-HaloTag7-His6 were commercially synthesized (General Bio). The plasmid pSNAPf-H2B was a gift from New England Biolabs & Ana Egana (NEB #N9186). The plasmid pCDNA3.1-mCherry-Vimentin was purchased from NEST Biotechnology Co.LTD. Primers for cloning were synthesized by General Bio in China. Fluorescence measurements were performed on an Agilent CARY Eclipse fluorescence spectrophotometer. Confocal images were performed on Laser Scanning Confocal Microscope (Andor iQ 3.2) with 60×oil-immersion objective lens (laser combination: 488 nm).

Design and construction of plasmids.

All plasmid constructions underwent validation through DNA sequencing.

Construction of the plasmid pET22b-SNAPtag-His6: the SNAP-tag sequence containing NheI and XhoI restriction sites was first amplified from the plasmid pCMV-pSNAPf (commercially synthesized). Subsequently, the plasmid pET22b-HaloTag7-His6 (commercially synthesized) was double-digested with NheI and XhoI restriction enzymes (TAKARA) to obtain the vector. The SNAP-tag sequence was then inserted into the vector using the One Step Cloning Kit (YEASEN), resulting in the construction of the pET22b-SNAPtag-His6 plasmid.

Construction of the plasmid pCMV-SNAPtag-mODC: using the pCMV-SNAPtag plasmid as a template, primers containing the mODC sequence and the BamHI restriction site were introduced to the C-terminus of the SNAP-tag through a two-step PCR process, resulting in the amplification of the SNAPtag-mODC sequence. The plasmid pCMV-pSNAPf (commercially synthesized) was then single-digested with BamHI restriction enzymes (TAKARA) to obtain

the vector. The SNAPtag-mODC sequence was then inserted into the vector using the One Step Cloning Kit (YEASEN), resulting in the construction of the pCMV-SNAPtag-mODC plasmid.

Construction of the plasmid pCMV-3HA-SNAPtag-mODC: the 3HA sequence containing EcoRI restriction site was first amplified from the pCDNA3.1-mCherry-Vimentin plasmid (NEST Biotechnology Co. Ltd). Subsequently, using the pCMV-SNAPtag plasmid as a template, primers containing the mODC sequence and the BamHI restriction site were introduced to the C-terminus of the SNAP-tag through a two-step PCR process, resulting in the amplification of the SNAPtag-mODC sequence. The plasmid pCMV-pSNAPf (commercially synthesized) was then double-digested with EcoRI and BamHI restriction enzymes (TAKARA) to obtain the vector. The 3HA and SNAPtag-mODC sequences were then inserted into the vector using the One Step Cloning Kit (YEASEN), resulting in the construction of the pCMV-3HA-SNAPtag-mODC plasmid.

Protein expression and purification.

The plasmid pET22b-SNAPtag-His6 was introduced into E.coli strain BL21 (DE3, TransGen Biotech) following standard transformation procedures. After transformation, the cells were plated onto selective agar plates containing Ampicillin (Amp). A single colony was then selected and inoculated into 5 mL of LB medium supplemented with Amp and incubated overnight at 37°C. The next day, the culture was diluted 1:100 into 350 mL of fresh LB medium containing Amp, and grown until the optical density at 600 nm (OD_{600}) reached 0.6-0.8. Protein expression was induced by the addition of 0.4 mM isopropyl β-D-1-thiogalactopyranoside (IPTG), and the cells were cultured at 19°C for 20 hours. After induction, the cells were harvested by centrifugation and resuspended in lysis buffer (20 mM PBS, 0.3 M NaCl, 1 mM PMSF, pH 7.4). The cell suspension was then disrupted by sonication, and the resulting debris was removed by centrifugation to obtain the supernatant. The target protein was purified from the lysate using Ni-NTA affinity chromatography (GE Healthcare) with a 250 mM imidazole buffer (20 mM PBS, 0.3 M NaCl, pH 7.4). Subsequently, the purified protein was concentrated by centrifugation through a 10 kDa ultrafiltration device (Millipore Corporation) and washed

with buffer lacking imidazole. The purified protein was stored at -80°C for long-term preservation. Protein purity was assessed by SDS-PAGE.

Fluorescence spectral measurements.

Fluorescence turning-on testing of BGAN-R probes for SNAP-tag. BGAN-R were prepared as described in previous work.[1,2] Stock solutions (5 mM) were prepared in DMSO and diluted to 4 μM in PBS buffer (20 mM, pH 7.4). The solution was divided into two groups: one group was added SNAP-tag protein (5 μM) and incubated at 37°C for 2 hours to prepare BGAN-R-SNAPtag conjugates, and the other group added an equal volume of PBS. Fluorescence emission from both groups was measured. Fluorescence spectra were recorded using an Agilent Cary Eclipse fluorescence spectrophotometer with an excitation wavelength of 450 nm.

Fluorescence turning-off testing of BGAN-R probes for protein degradation. BGAN-R (5 mM) was diluted to 4 μM in PBS buffer (20 mM, pH 7.4), and SNAP-tag protein (5 μM) was added and incubated at 37°C for 2 h for protein labeling. BGAN-R-SNAPtag conjugates were digested with trypsin (4 μM) at 37°C for 2 h, and fluorescence emission was measured. Fluorescence spectra were recorded using an Agilent Cary Eclipse fluorescence spectrophotometer with an excitation wavelength of 450 nm.

Fluorescence spectra testing of AN-R probes. BGAN-R were prepared as described in previous work.[1,2] AN-R (5 mM) was diluted to 10 μM in different in different solvents. Fluorescence spectra were recorded using an Agilent Cary Eclipse fluorescence spectrophotometer with an excitation wavelength of 450 nm.

Fluorescence kinetic study of BGAN-8C probe for SNAP-tag degradation.

The BGAN-8C-SNAPtag conjugate was incubated with 4 μM trypsin in PBS buffer at 37°C. The fluorescence intensity at 530 nm (excitation at 450 nm) was monitored over time. Fluorescence kinetics were recorded using an Agilent Cary Eclipse fluorescence spectrophotometer.

In vitro protein degradation analysis by SDS-PAGE.

The SNAP-tag protein was diluted to 5 μM in PBS buffer and incubated with 4 μM trypsin at 37°C. The reaction mixture was heated at 100°C for 10 minutes and then analyzed by 15% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The gel was stained with Coomassie Brilliant Blue (CBB).

Dynamic Light Scattering (DLS) size determination.

DLS experiments were carried out with Malvern Zetasizer NanoZS90. All measurements with the probe concentration of 5 μM were repeated for three times at 25 °C. Samples were prepared in 20 mM PBS, pH 7.4.

Cell culture.

HeLa (ATCC: CCL-2) and HKE293T (ATCC: CRL3216) cells were purchased from Cell Bank of Type Culture Collection of Chinese Academy of Sciences. Cells were grown in Dulbecco's modified Eagle's medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS, Hyclone) and 1% antibiotics (Hyclone), which were cultured in the humidified atmosphere of 5% CO_2 at 37°C. Before imaging, Cells were seeded on glass bottom cell culture dish (Nest, polystyrene, Φ 15 mm) for 24 h to reach 60-80% confluency. The next day, cells were transfected with 0.5 μg of DNA using polyethyleneimine (PEI, Polyscience) according to the manufacturer's protocol. Cell imaging was performed 24-48 hours after transient transfection.

Western blot analyses.

HEK293T cells were transfected with pCMV-3HA-SNAPtag-mODC using PEI (Polyscience) and incubated at 37°C for 48 hours. Cells were then incubated with 100 μg/ml CHX (MCE) for 0 and 3 hours, and 10 μM MG132 (MCE) for 3 hours. Subsequently, cells were harvested and lysed in RIPA lysis buffer containing 1× protease inhibitor cocktail (MCE) and 1 mM phenylmethylsulfonyl fluoride (PMSF, Servicebio) for 30 minutes. Proteins were heated at 100 °C for 10 minutes. Protein extracts were separated by SDS-PAGE with a gel

concentration of 15% and then blotted onto PVDF membranes by wet transfer. The membranes were then blocked with 5% skim milk in TBS containing 0.1% Tween-20 (TBST) at room temperature for 4-6 hours. Primary antibodies against HA tag (Beyotime) and internal control Vinculin (Abclonal) were diluted in commercial antibody diluent and incubated with the membrane overnight at 4°C. After washing three times with TBST, the membrane was incubated with species-specific horseradish peroxidase (HRP)-conjugated secondary antibodies for 1.5 hours at room temperature. Protein bands were visualized using an enhanced chemiluminescence (ECL) detection system, and signals were captured using a chemiluminescence imaging system.

Confocal fluorescence imaging.

Live-cell confocal imaging of H2B-SNAP protein labeled with BGAN-R. Hela cells transfected with pSNAPf-H2B (NEB #N9186) were imaged after adding the BGAN-R (5 μM) and incubating for 2 h, and then washed three times with DMEM (Gibco). Excitation channel, λex: 488 nm, collection wavelength: 500-550 nm.

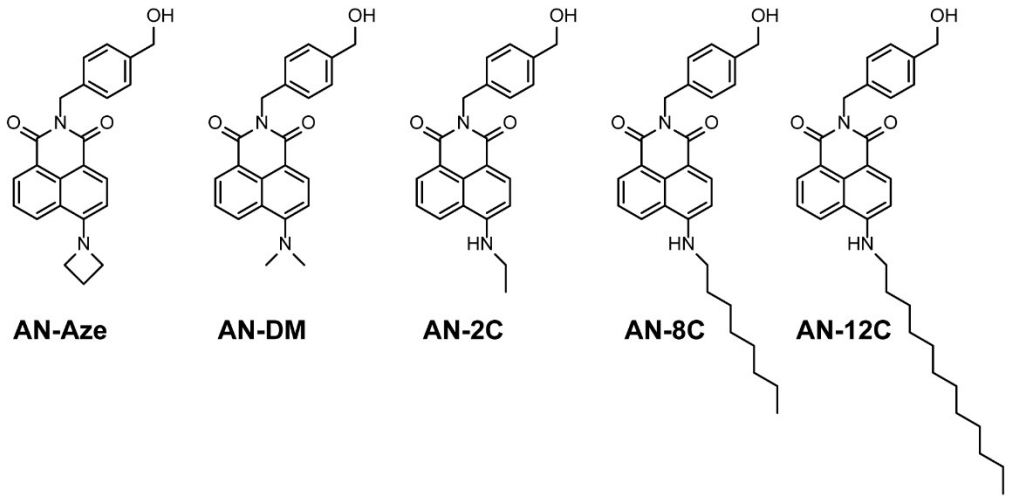
Fluorescence tracking imaging of SNAP-tag protein degradation in HEK293T cells with BGAN-8C. HEK293T cells transfected with pCMV-SNAPtag and pCMV-SNAPtag-mODC plasmids were incubated with BGAN-8C at 37°C for 2 hours, and then washed 3 times with DMEM. Subsequently, time-lapse imaging was performed using a confocal laser scanning microscope. Imaging was conducted with a 30-minute interval over a total duration of 4 hours. All images were captured using an excitation wavelength of 488 nm and collecting emission within the 500-550 nm range.

Fluorescence tracking imaging of SNAP-tag protein degradation in HEK293T cells with BGAN-8C in the presence of MG132. HEK293T cells transfected with pCMV-SNAPtag-mODC plasmid was incubated with BGAN-8C at 37°C for 2 hours, and then washed 3 times with DMEM. Before imaging, the medium was replaced with serum-containing DMEM medium containing 10 μM MG132 (MCE). Subsequently, time-lapse imaging was performed using a confocal laser scanning microscope. Imaging was conducted with a 30-minute interval

over a total duration of 4 hours. All images were captured using an excitation wavelength of 488 nm and collecting emission within the 500-550 nm range.

Data processing and analysis.

The generation of all spectral curves in this study was processed using Origin 2018 software. Fluorescence intensity data of live cell imaging were obtained by the built-in analysis software of the imaging system. GraphPad Prism 8 and Origin 2018 software were used to analyze the average intensity and draw intensity trend lines. Western blot image grayscale data were analyzed using ImageJ Fiji.



Scheme S1. Structure of control AN-R compounds.

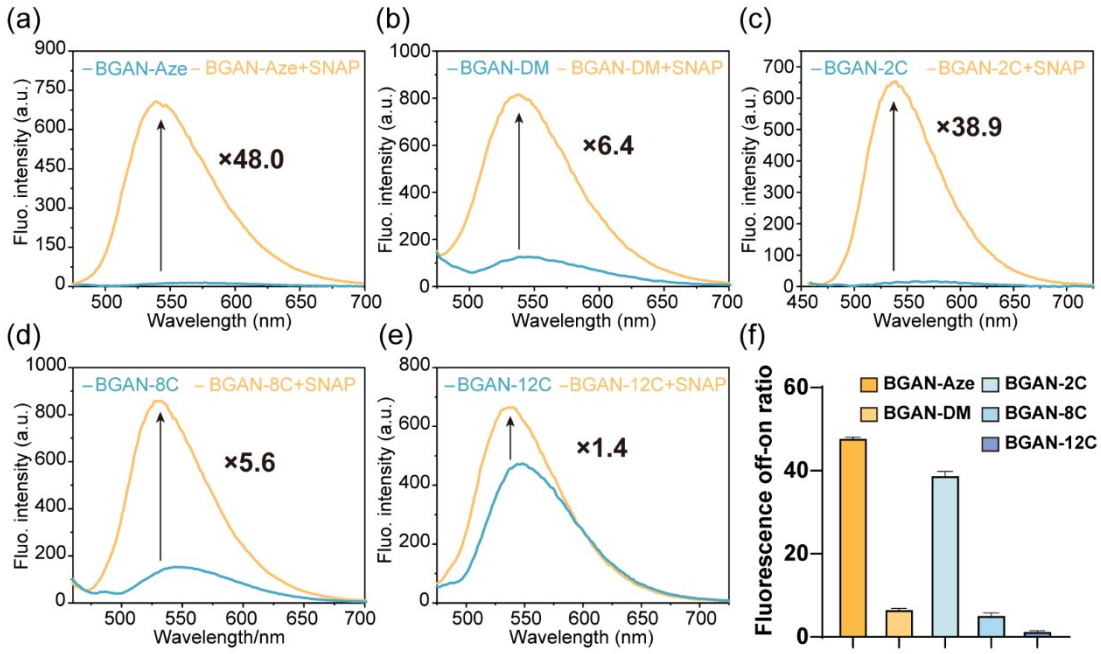


Figure S1. (a-e) Fluorescence spectra of the BGAN-R probes (4 μM) before and after binding to SNAP-tag (5 μM). (f) The average enhancement factor of BGAN-R upon reaction with excess SNAP-tag protein in PBS.

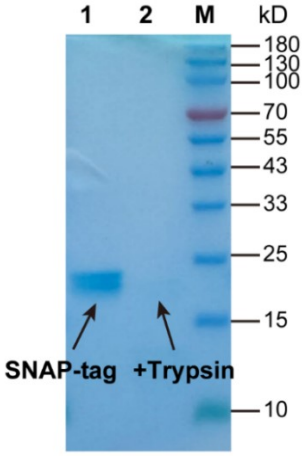


Figure S2. SDS-PAGE analysis of 5 μM SNAP-tag complexes in the presence or absence of 4 μM trypsin. The gel was stained with Coomassie Brilliant Blue (CBB).

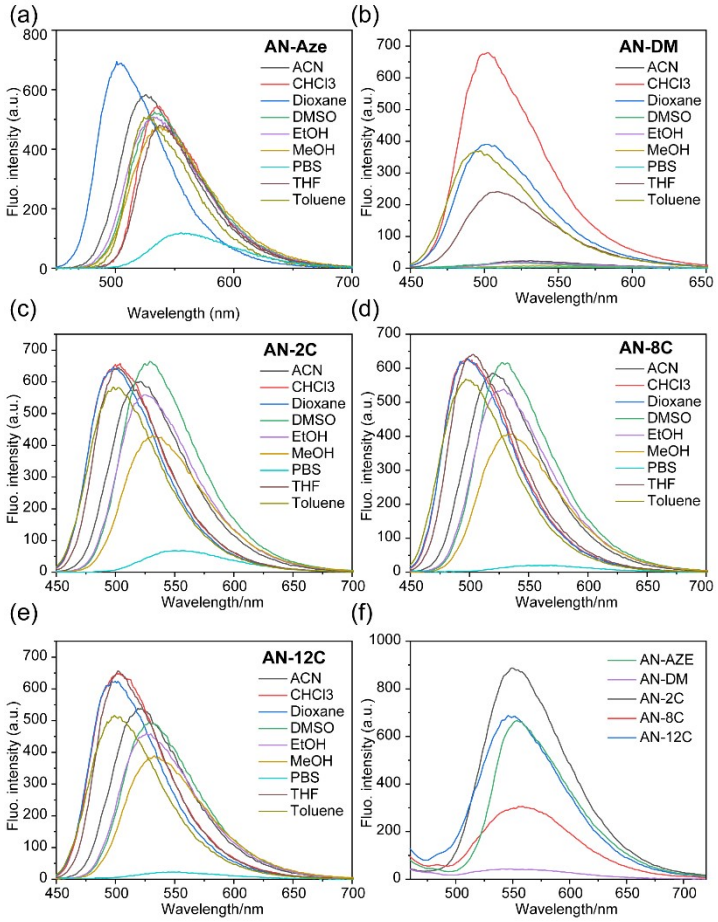


Figure S3. (a-e) Fluorescence spectra of AN-Aze (a), AN-DM (b), AN-2C (c), AN-8C (d), and AN-12C (e) in different solvents. [dyes] = 10 μM. (f) Fluorescence spectra of AN-R in PBS buffer. [dyes] = 10 μM. λ_{ex}: 450 nm.

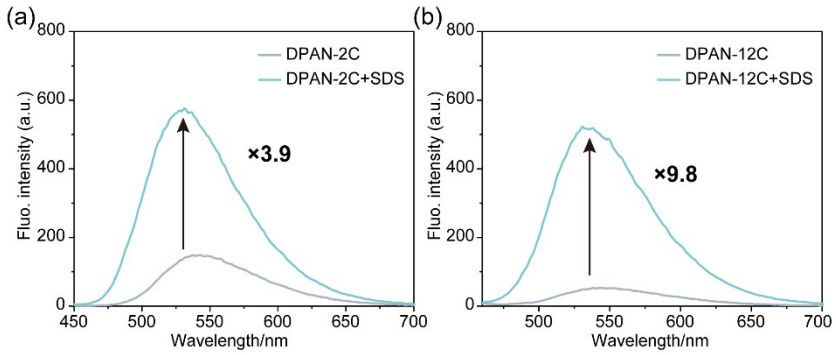


Figure S4. (a-b) Fluorescence spectra of DPAN-2C (a), and DPAN-12C (b) conjugates in the absence and presence of 10 mM SDS. λ_{ex}: 450 nm.

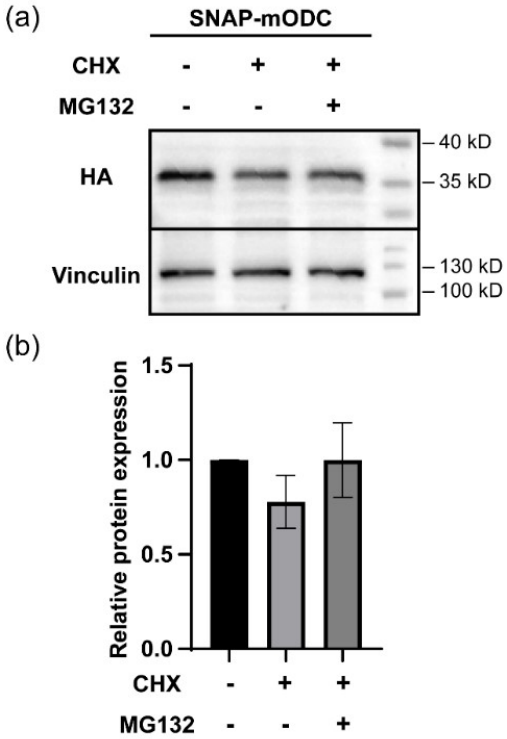


Figure S5. (a) Western blot analysis of 3HA-SNAPtag-mODC degradation. HEK293T cells were treated with or without 100 μg/mL CHX and 10 μM MG132. Anti-HA or anti-Vinculin antibody were used as primary antibodies. The exposure times for anti-HA and anti-actin were 4 seconds. (b) Relative protein levels were expressed as fold of control after normalization to vinculin. The molecular weight of 3HA-SNAPtag-mODC is 35.5 kD, and the internal reference antibody Vinculin is 124 kD.

Table S1. Optical Properties of AN-R in SNAP-tag and degradation products.

Compounds		λ_{em}[a]	Φ[b]	Φ_{SNAP}/Φ_{AN}[c]	$F_{SNAP}/F_{DP\text{-}AN}$[d]
Aze	SNAP-AN-Aze	541	0.39	1.56	1.5
	DP-AN-Aze	545	-		
	AN-Aze	555	0.25		
DM	SNAP-AN-DM	537	0.028	1.33	2.0
	DP-AN-DM	538	-		
	AN-DM	548	0.021		
2C	SNAP-AN-2C	539	0.367	1.28	1.5
	DP-AN-2C	541	-		
	AN-2C	550	0.286		
8C	SNAP-AN-8C	530	0.275	3.16	6.0
	DP-AN-8C	532	-		
	AN-8C	555	0.087		

[a] Fluorescence emission of AN-R in PBS buffer, SNAP-tag and degradation products. [b] Quantum yields of AN-R in PBS buffer, and SNAP-tag. [c] Ratio of the quantum yields of AN-R in PBS buffer and SNAP-tag. [d] Ratio of AN-R fluorescence intensity before and after SNAP-tag protein degradation.

Table S2. Amino acid sequences and molecular weights of the linker regions in SNAP and SNAP-mODC proteins.

	SNAP Protein squence	MW (Da)
SNAP	MASDIGAPAFKSVQTGEFTMDKDCEMKRTTLDSP LGKLELSGCEQGLHRIIFLGKGTSAADAVEVPAPA AVLGGPEPLMQATAWLNAYFHQPEAIEEFPVPAL HHPVFQQESFTRQVLWKLLKVVKFGEVISYSHLA ALAGNPAATAAVKTALSGNPVPILIPCHRVVQGDL DVGGYEGGLAVKEWLLAHEGHRLGKPGLGPAGG SAFKLEHHHHHH*	23170
	SNAP-mODC Protein squence	MW (Da)
SNAP-mODC	MGGRRVRWEVYISRALWLTREPTAYWLIEINTTH YRETQAWYRARIVASDIGAPAFKSVQTGEFTMDK DCEMKRTTLDSPLGKLELSGCEQGLHRIIFLGKGTS AADAVEVPAPAAVLGGPEPLMQATAWLNAYFHQ PEAIEEFPVPALHHPVFQQESFTRQVLWKLLKVVK FGEVISYSHLAALAGNPAATAAVKTALSGNPVPILI PCHRVVQGDLDVGGYEGGLAVKEWLLAHEGHRL GKPGLGPAGGHGFPPEVEEQDDGTLPMSCAQESG MDRHPAACASARINV*	31530
	3HA-SNAP-mODC Protein squence	
3HA-SNAP-mODC	MGGRRVRWEVYISRALWLTREPTAYWLIEINTTH YRETQAWYRARIVASDIGAPAFKSVQTGEATMEY PYDVPDYATYPYDVPDYASYPYDVPDYAEFTMD KDCEMKRTTLDSPLGKLELSGCEQGLHRIIFLGKG TSAADAVEVPAPAAVLGGPEPLMQATAWLNAYF HQPEAIEEFPVPALHHPVFQQESFTRQVLWKLLKV VKFGEVISYSHLAALAGNPAATAAVKTALSGNPV PILIPCHRVVQGDLDVGGYEGGLAVKEWLLAHEG HRLGKPGLGPAGGHGFPPEVEEQDDGTLPMSCAQ ESGMDRHPAACASARINV*	35530

Reference

1 S. Leng, Q. Qiao, L. Miao, W. Deng, J. Cui and Z. Xu, *Chem. Commun.*, 2017, **53**, 6448–6451.

2 Q. Qiao, W. Liu, W. Chi, J. Chen, W. Zhou, N. Xu, J. Li, X. Fang, Y. Tao, Y. Zhang, Y. Chen, L. Miao, X. Liu and Z. Xu, *Aggregate*, 2023, **4**, e258.