Supplementary Information for

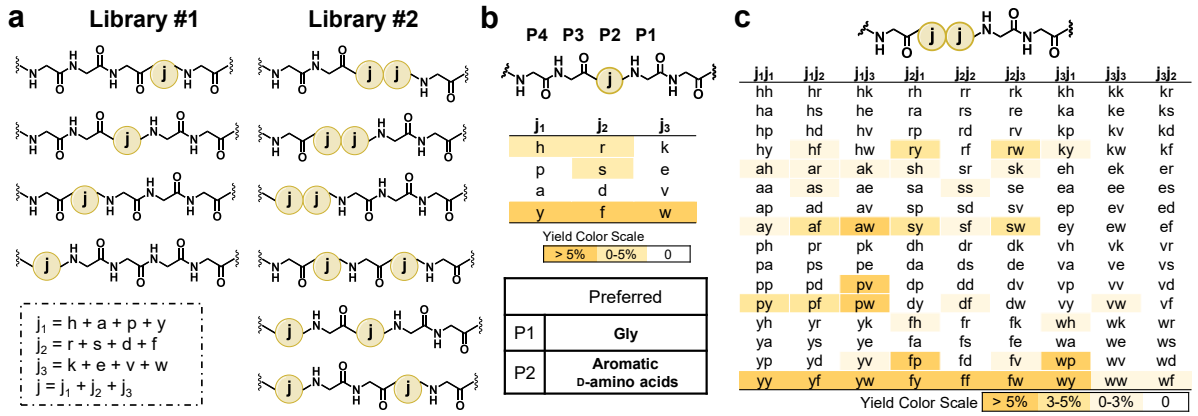
Transcending Stereochemical Boundaries: Ambidextrous Cleavage of D- and L-Peptide Enantiomers by Natural Eukaryotic Proteases

Mengjiao Li, Kai Chen, Wanyang Zhang, Jianyi Han, Mengzhun Guo, Xinxin Zhu, Jia Zheng, Jing Huang*, Tian Li*, Bobo Dang*

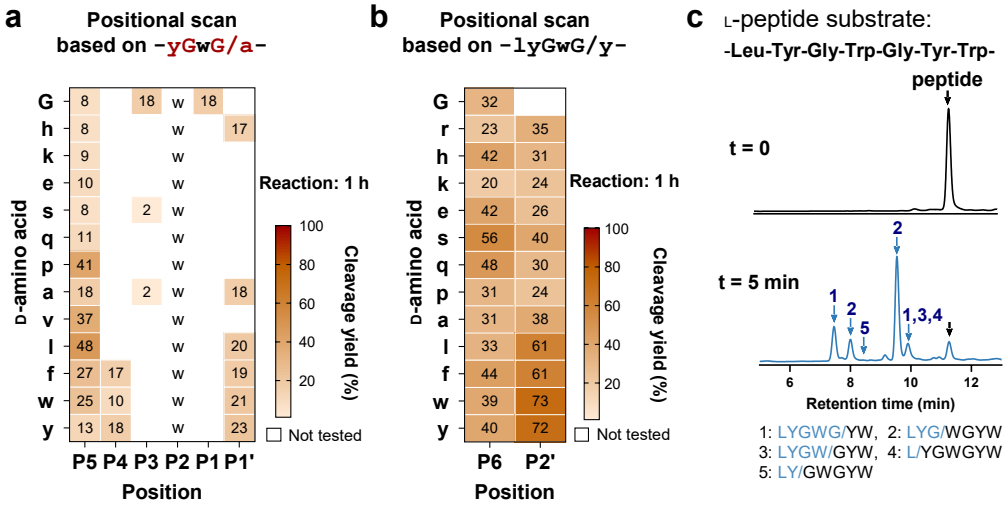
*Corresponding authors: dangbobo@westlake.edu.cn, litian32@westlake.edu.cn, huangjing@westlake.edu.cn

This PDF file includes:

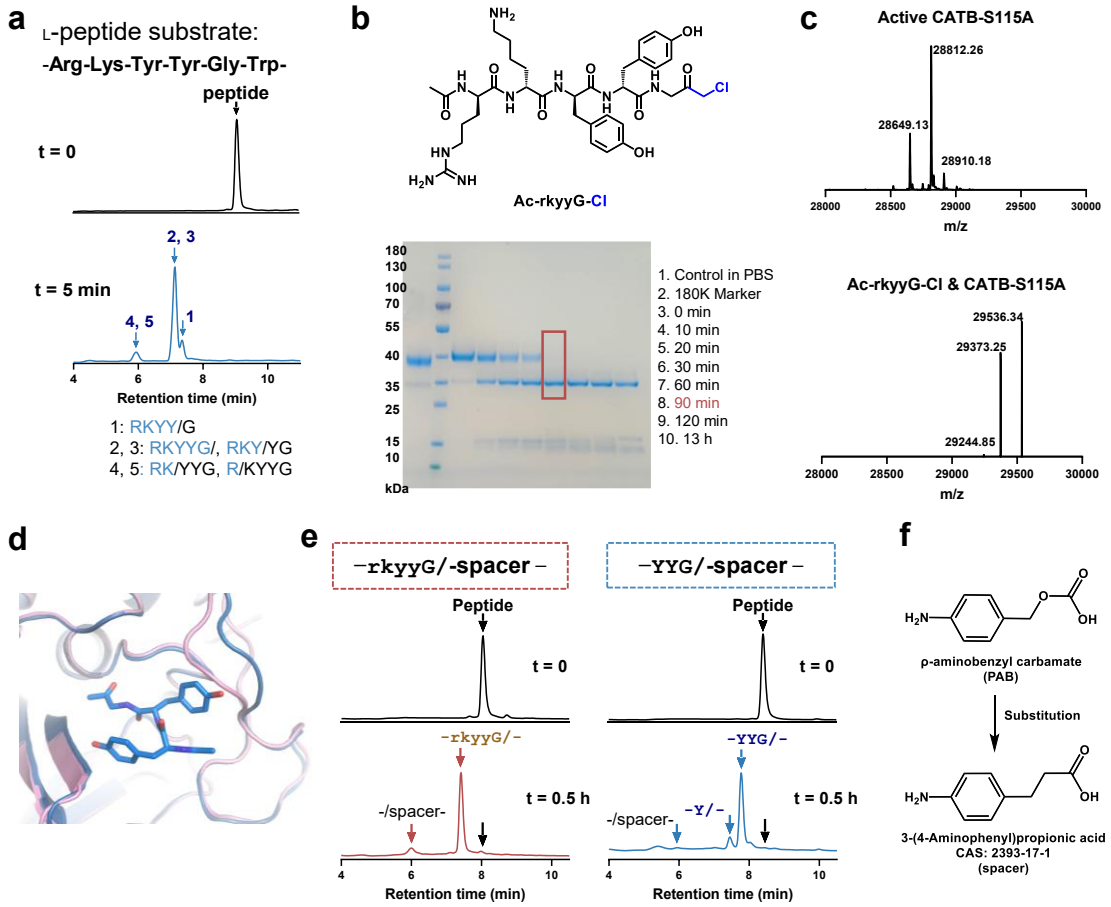
Supplementary Figures



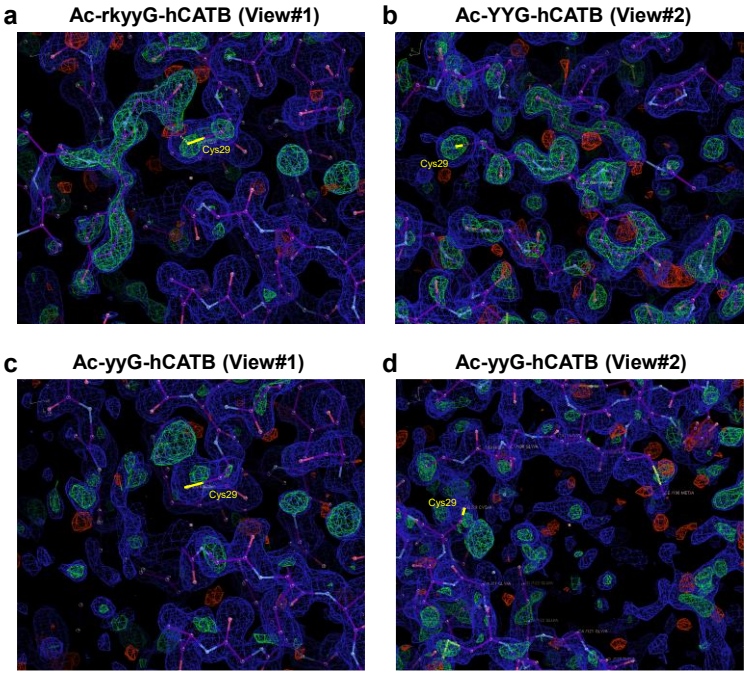
Supplementary Fig. S1. The reactions of hCATB with D-peptide library. a, The design of the combinatorial D-tetrapeptide **Library #1, #2** for hCATB screening. **j** denotes mixed amino acids while lowercase letters represent D-amino acids. **b,c,** Initial screening results of D-peptide substrates of hCATB using combinatorial D-tetrapeptide −GGjGG− (**b**) and −Gjj GG− (**c**).



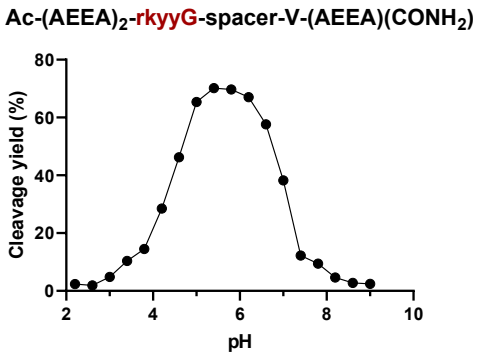
Supplementary Fig. S2. Papain recognizes D-peptide substrate with high sequence specificity. a, Site saturation mutagenesis screening of D-amino acids at P5-P1' based on the peptide −yGwGa− to identify optimal D-peptide substrate following a 1-hour papain treatment. **Fig. 2E** in the main text reports results from a 36-hour papain treatment. **b,** Site saturation mutagenesis screening of D-amino acids at P6 and P2' based on the candidate peptide −lyGwGy−. The slash represents the cleavage site; numbers represent the percentage of cleavage yield at 37°C; $n = 2$ independent replicates. **c,** The proteolytic activity of papain on the corresponding L-peptide substrate −LYGWGYW−. The slash represents the cleavage site; cleaved fragments are labeled as numbers 1-5. **(a-c)** The concentration of papain was 235 nM, and the substrates concentration was 40 μM.



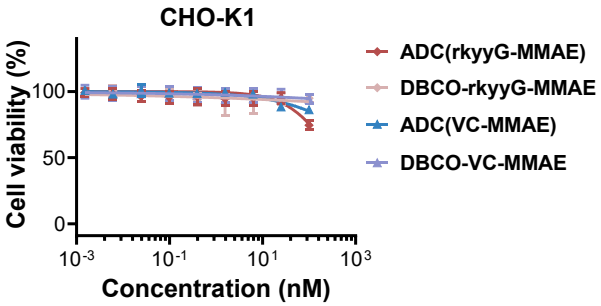
Supplementary Fig. S3. The reactions of hCATB with different substrates. a, The proteolytic activity of hCATB on the corresponding L-peptide substrate −RKYYGW−. Cleaved fragments are labeled as numbers 1-5. **b,** Upper panel: the structure of Ac-rkyyG-Cl, covalently targeting the catalytic Cys29 of hCATB. Lower panel: the activation of hCATB-S115A in MES buffer at room temperature was confirmed using SDS-PAGE. **c,** Upper panel: Deconvoluted LC-MS of active hCATB-S115A reveals distinct peaks corresponding to various cleavages of the C-propeptide "QYWEKI". Lower panel: Deconvoluted LC-MS confirms that Ac-rkyyG-Cl covalently binds to hCATB-S115A. **d,** The loop Thr120-Gly123 of hCATB went through a conformational change to accommodate L-peptide (blue) upon binding, compared with D-peptide bound structure (pink). **e,** hCATB recognizes −rkyyG− and −YYG−, all achieving complete cleavage within 0.5 hours. **f,** The structure of PAB and the substituted spacer for screening. **(a,e)** The concentration of hCATB was 20 nM, and the substrate concentration was 40 μM.



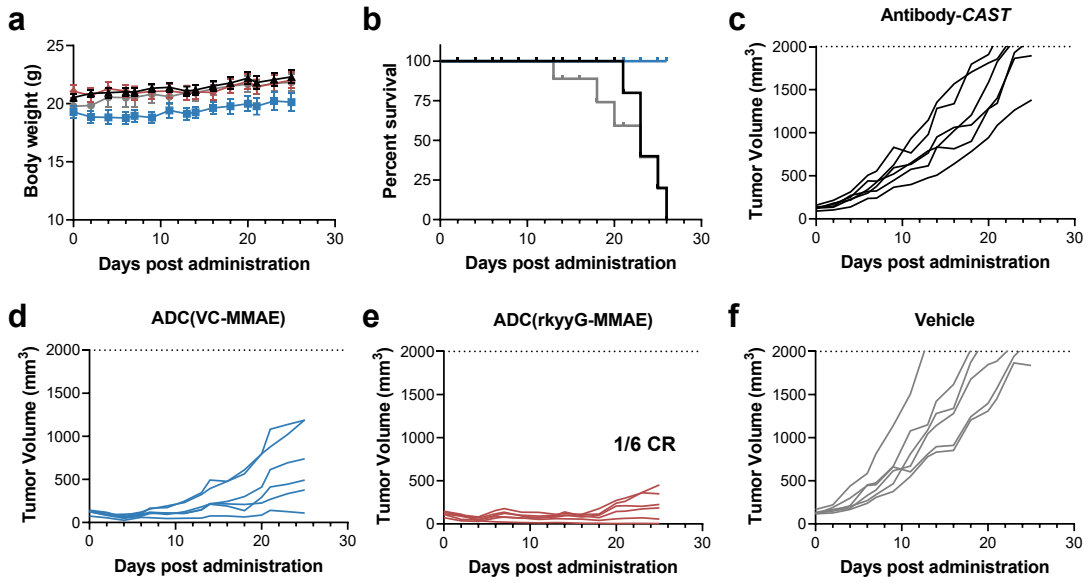
Supplementary Fig. S4. Comparison of local electron density maps for peptide substrates in complex with hCATB. a,c, Identical views of Ac-rkyyG-hCATB and Ac-yyG-hCATB，showing clear electron density for Ac-rkyyG, but not Ac-yyG. **b,d,** Identical views of Ac-YYG-hCATB and Ac-yyG-hCATB, showing clear electron density for Ac-YYG, but not Ac-yyG.



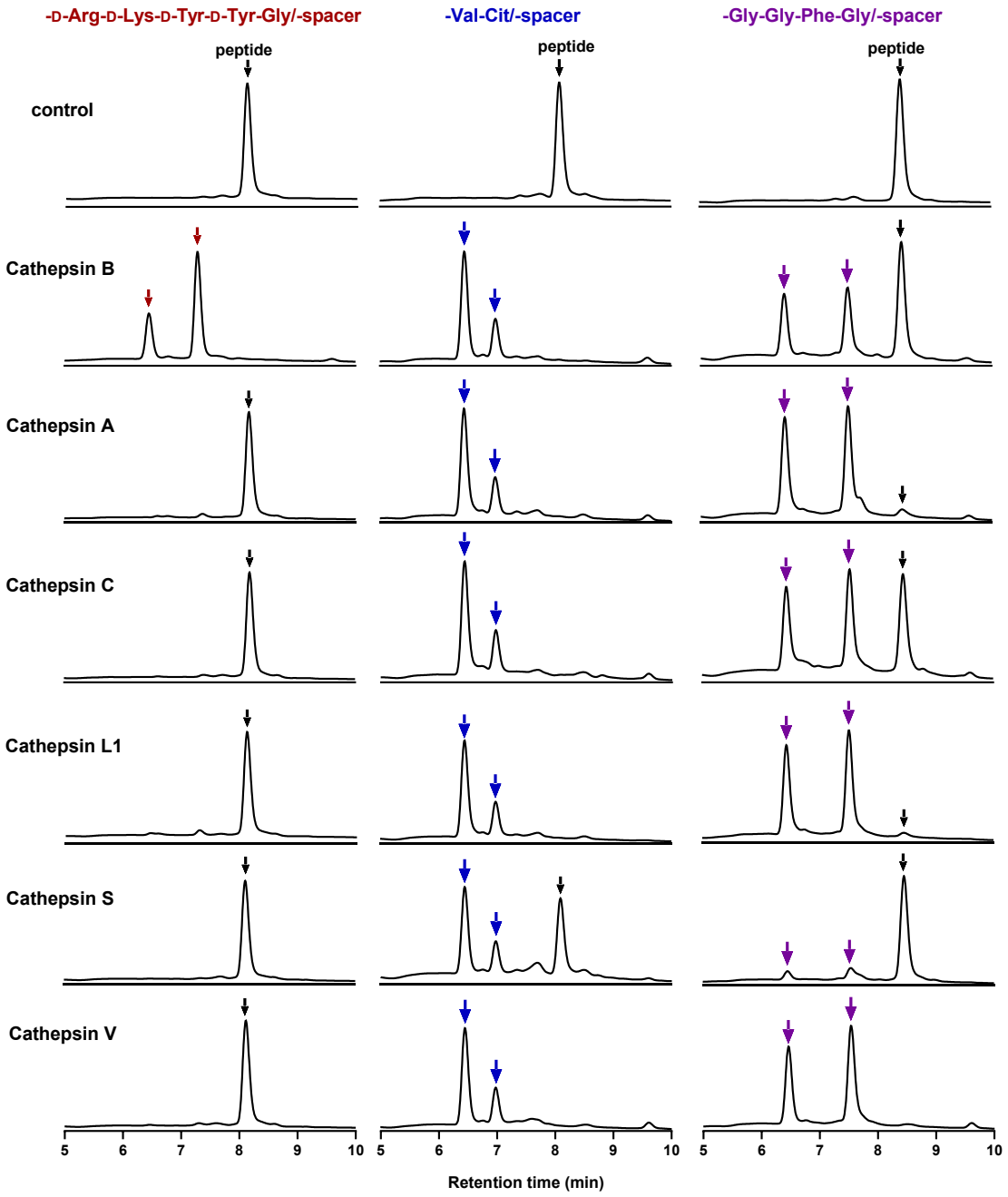
Supplementary Fig. S5. pH-dependent D-peptide cleavage preference of hCATB. Following the methodology described in PMID: 35981509[51], hCATB exhibited pH-dependent endopeptidase activity toward the D-peptide substrate Ac-AEEA-rkyyG-spacer-Val-AEEA($CONH_2$).



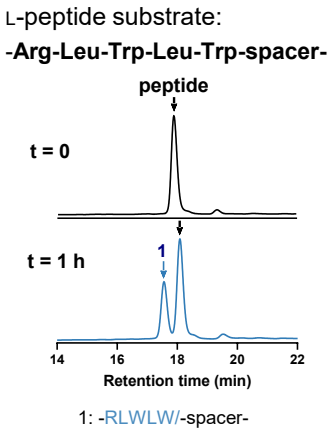
Supplementary Fig. S6. Cytotoxicity of the CLDN18.2 ADC in CHO-K1 cells. Data are presented as means ± SD (n = 3).



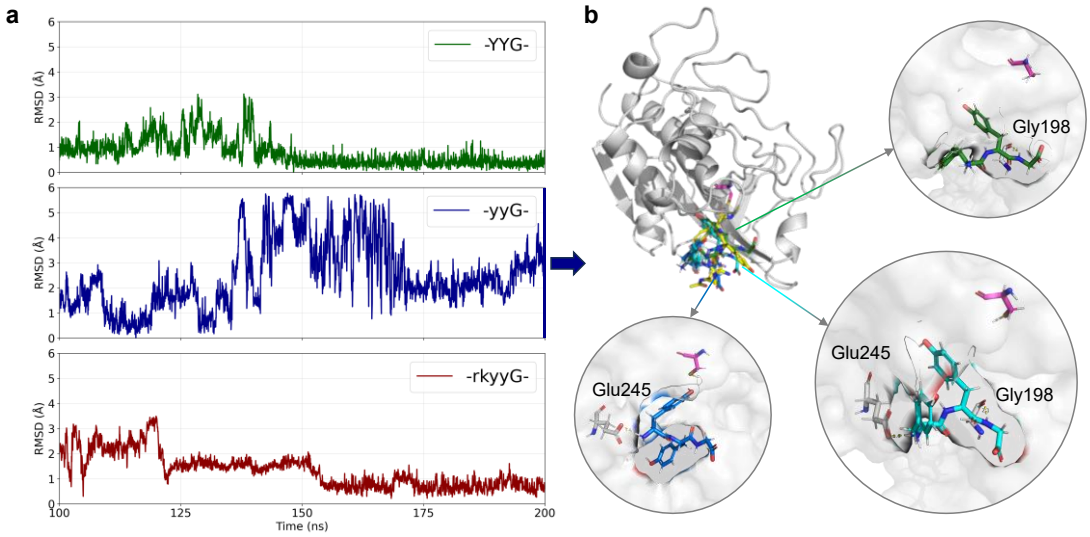
Supplementary Fig. S7. In vivo efficacy of CLDN18.2 ADCs. a, Tumor weights of ADCs in the NUGC-4 xenograft model were determined until day 25. Data points represent mean values, with error bars indicating SEM (n = 6). **b,** Survival percentages (n = 6) during the treatment and observation period. The mice were euthanized when the volume of the subcutaneous xenografts exceeded 2000 mm^3. **c-f,** Individual tumor volume growth curves for mice received (**c**) Antibody-*CAST*, (**d**) ADC(VC-MMAE), (**e**) ADC(rkyyG-MMAE) and (**f**) Vehicle control. CR, complete response.



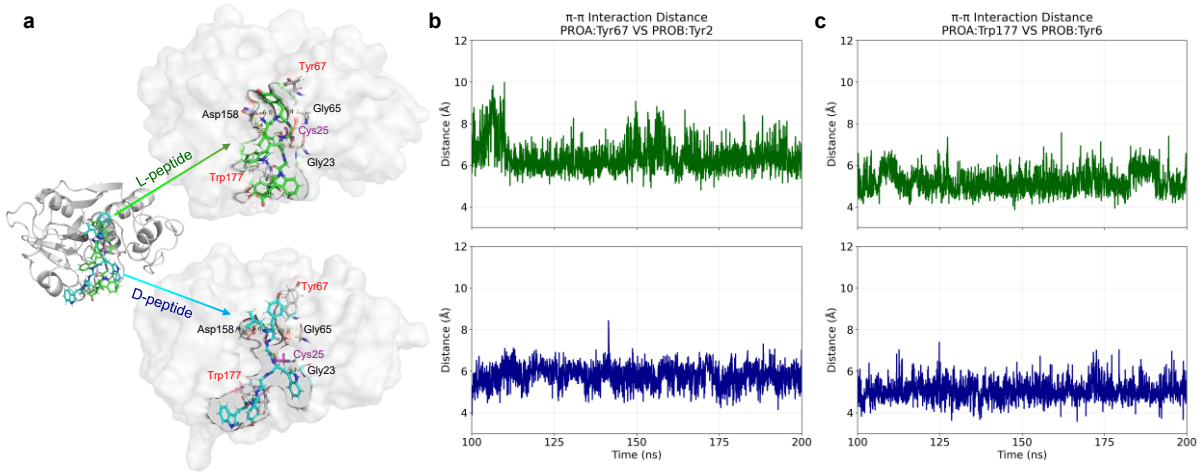
Supplementary Fig. S8. The cleavage activity of different peptides towards cathepsin family proteins. The cleavage specificity of D-peptide substrate −rkyyG−, commercial ADC linker −VC−, and commercial ADC linker −GGFG− against cathepsin proteases is presented. Black arrows indicate the raw materials, whereas colored arrows denote the cleaved products. Peptides: 40 μM; Cathepsin B: 0.86 μg/mL (20 nM); Cathepsin A: 7.5 μg/mL; Cathepsin C: 7.5 μg/mL; Cathepsin L: 3.75 μg/mL; Cathepsin S: 15μg/mL; Cathepsin V: 3.75 μg/mL; All reactions were processed at 37℃ for 12 hours. The cleavage percentages are shown in **Supplementary Table S30.**



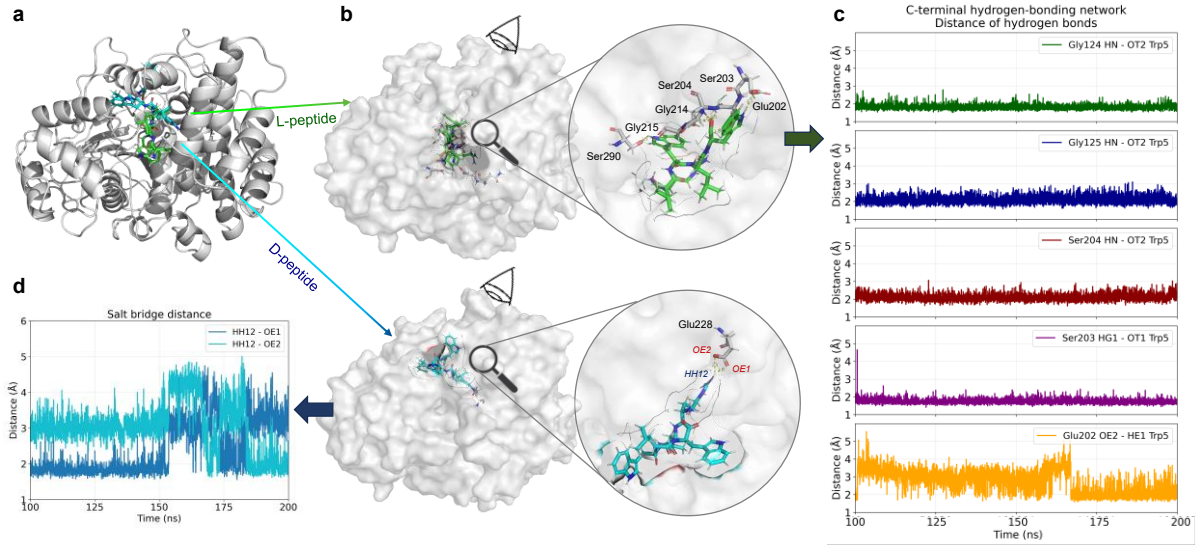
Supplementary Fig. S9. The proteolytic activity of mCES1c on the corresponding L-peptide substrate –RLWLW–. Cleaved fragment is labeled as number **1**. The concentration of mCES1c was 33.7 nM, and the substrate concentration was 40 μM.



Supplementary Fig. S10. The L-tripeptide (-YYG-) binds stably with hCATB, while the D-tripeptide (-yyG-) exhibits heterogenous binding conformations. a, RMSDs of the L-tripeptide (green), D-tripeptide (blue), and D-pentapeptide (red) over the last 100 ns of MD simulations, showing stable binding for -YYG- and -rkyyG-, but not-yyG-. **b**, Zoomed-in views of the D-tripeptide in the bound state. Multiple binding conformations are observed, primarily stabilized by interactions with Glu245 and Gly198.

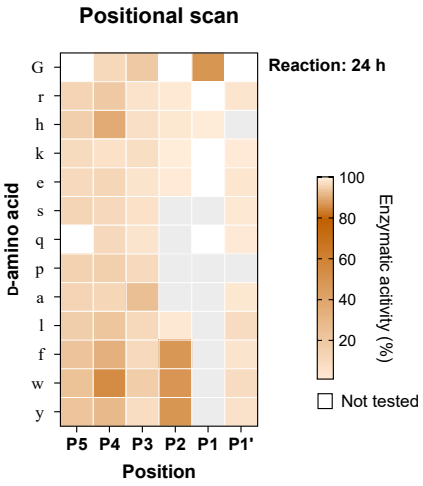


Supplementary Fig. S11. Computational modeling of substrate recognition of D-peptide (-lyGwGyw-) and L-peptide (-LYGWGYW-) by papain. a, Binding pockets and key contact residues involved in peptide recognition. **b,** Center-of-mass (CoM) distance between the aromatic rings of Tyr67 in papain and Tyr2 in the peptide. Tyr67 engages in T-shaped π–π stacking with L-Tyr2, whereas it adopts a slipped-parallel π–π stacking geometry with D-Tyr2. **c,** CoM distance between the aromatic rings of Trp177 in papain and Tyr6 in the peptide, indicating stable π–π stacking interactions in both systems.



Supplementary Fig. S12. Computational modeling of substrate recognition of L-peptide (-RLWLW-) and D-peptide (-rlwlw-) by mCES1c. a, Superposition of the L- and D-peptide within the buried pocket of mCES1c. **b,** Distinct regions of the pocket are occupied by the chiral peptides, with zoomed-in views of their binding modes and key contact residues involved in peptide recognition. **c,** Distances of the hydrogen bonds formed between Trp5 in the L-peptide and the C-terminal residues of mCES1c over the last 100 ns of MD simulations. **d.**

Distance of the salt bridge between the first residue D-Arg of the D-peptide and Glu228 in mCES1c over the last 100 ns of MD simulations.



Supplementary Fig. S13. Positional scans of D-amino acids for cathepsin L. Site saturation mutagenesis screening of D-amino acids across the P5 to P1' positions. The parental D-peptide substrate is -rkyyG-. The concentration of in-house-expressed hCATL was 20 μg/mL, and the peptide concentration was 40 μM. Detailed cleavage yields are provided in **Supplementary Tables S35,36.**

Supplementary Materials and Methods

1. General information

Chemicals

All chemicals were purchased from commercial vendors and used without further purification. Fmoc-Rink amide resin was purchased from Hecheng Technology (Tianjin, China). 1-[Bis(dimethylamino)methylen]-5-chlorobenzotriazolium 3-oxide hexafluorophosphate (HCTU), Fmoc-Gly-OH, Fmoc-L/D-Ala-OH, Fmoc-L/D-Leu-OH, Fmoc-L/D-Val-OH, Fmoc-L/D-Ser(^{t}Bu)-OH, Fmoc-L/D-Lys(Boc)-OH, Fmoc-L/D-Arg(Pbf)-OH, Fmoc-L/D-Phe-OH, Fmoc-L/D-Trp(Boc)-OH, Fmoc-L/D-His(Boc)-OH, Fmoc-L/D-Tyr(^{t}Bu)-OH, Fmoc-L/D-Pro-OH, Fmoc-L/D-Glu(O^{t}Bu)-OH, Fmoc-D-Gln(Trt)-OH, Fmoc-D-Asn(Trt)-OH, Fmoc-D-Asp(O^{t}Bu)-OH, Fmoc-D-Thr(^{t}Bu)-OH, Fmoc-D-Met-OH and Fmoc-D-Ile-OH, were purchased from GL Biochem and enantiomeric purity of each D-amino acid is listed in Supplementary Table S1. HPLC-grade trifluoroacetic acid (TFA), triisopropylsilane (TIPS), and dithiothreitol (DTT) were purchased from Bidepharm. N, N-dimethylformamide (DMF), dichloromethane (DCM), 4-methyl-piperidine diethyl (PIP), ether, and HPLC-grade acetonitrile were obtained from Energy Chemical. *Solvent A1: 0.1% TFA in H_2O. Solvent B1: 0.08% TFA in acetonitrile. Solvent A2: 0.1% FA in H_2O. Solvent B2: 0.08% FA in acetonitrile.*

Abbreviations

Ac: Acetyl
AEEA: Amino Ethyl Ethanol Amine
AEEA2: AEEA-AEEA
VC: Val-Cit
MES: 2-Morpholinoethanesulphonic acid monohydrate
PBS: Phosphate-buffered saline
NMM: N-Methylmorpholine

2. Standard enzymatic reaction conditions and screnning workfolw

Papain (EC 3.4.22.2)

To a solution of peptide (0.8 μL of a 4 mM stock solution in 10% DMSO/H_2O, final concentration 0.08 mM) in 10 mM Tris-HCl buffer (pH 6.2, containing 4 mM EDTA and 5 mM L-cysteine, 38.8 μL), papain (Macklin, P6321, 0.8 μL of a 275 μg/mL stock solution in Tris-HCl buffer, final concentration 5.5 μg/mL (235 nM), approximately 1 μmol: 13.75 U) was added. The mixture was vortexed and incubated at 37°C. At specified time intervals, the mixture was boiled at 95°C for 10 minutes to terminate the reactions. The crude reaction mixture was then centrifuged and analyzed by LC-MS to determine the reaction yield.

Human Cathepsin B (EC 3.4.22.1)

To a solution of peptide (0.4 μL of a 4 mM stock solution in 10% DMSO/H2O, resulting in a final concentration of 0.04 mM) in 25 mM MES buffer (pH 5.5, containing 1 mM EDTA and 5 mM DTT, 39.2 μL), human cathepsin B (SinoBiological, 10483-H08H, 0.4 μL of a 2 μM stock solution in PBS, resulting in a final concentration of 0.86 μg/mL (20 nM), approximately 1 μmol: 0.046 U) was added subsequently. The mixture was vortexed and then incubated at 37°C. At defined time points, the mixture was boiled at 95°C for 10 minutes to quench the reaction. The crude reaction mixture was then centrifuged and analyzed by LC-MS to determine the reaction yield.

Mouse Carboxylesterase 1c (EC 3.1.1.1)

To a solution of peptide (0.4 μL of 4 mM stock solution in 10% DMSO/H_2O, 0.04 mM final concentration) in PBS buffer (pH 7.4, 38.8 μL), mCES1c (MCE, HY-P71802, 0.8 μL of 100 μg/mL stock solution in water, 2 μg/mL (33.7 nM) final concentration, no unit tests) was added subsequently. The mixture was vortexed and then incubated at 37 °C. At defined time points, the mixture was boiled at 95°C for 10 minutes to quench the reactions, then the crude reaction mixture was centrifuged and analyzed by LC-MS to determine the reaction yield.

Human Cathepsin A (Sinobiological, 10482-H08H, no specific activity)

The activation buffer consists of 25 mM MES, and 5 mM DTT, at pH 6.0. The assay buffer comprises 25 mM MES at pH 5.5, 1 mM EDTA, and 5 mM DTT. RhCathepsin A was diluted to 200 μg/mL in the activation buffer. RhCathepsin L and the activation buffer were added to rhCathepsin A to achieve final concentrations of 20 μg/mL rhCathepsin L and 100 μg/mL rhCathepsin A. The mixture was incubated at room temperature for 1 hour. The activated rhCathepsin A was then diluted to 7.5 μg/mL in the assay buffer, with a total volume of 50 μL, and the substrate was diluted to 40 μM, with a volume of 0.5 μL. The protease and substrate were incubated at 37°C for 12 hours or longer. A portion of the mixture was taken for LC-MS monitoring.

Human Cathepsin C (Sinobiological, 10484-H08H, >200 pmols/min/ug)

The activation buffer consists of 25 mM MES, and 5 mM DTT, at pH 6.0. The assay buffer comprises 25 mM MES, 50 mM NaCl, 1 mM EDTA, and 5 mM DTT, at pH 6.0. RhCathepsin C was diluted to 200 μg/mL in the activation buffer. RhCathepsin L and the activation buffer were added to rhCathepsin C to achieve final concentrations of 20 μg/mL rhCathepsin L and 100 μg/mL rhCathepsin C. The compounds were incubated at room temperature for 1 hour. The activated rhCathepsin C was then diluted to 7.5 μg/mL in the assay buffer, with a total volume of 50 μL, and the substrate was diluted to 40 μM in 0.5 μL. The protease and substrate were incubated at 37°C for 12 hours or longer. A portion of the mixture was subsequently taken for LC-MS monitoring.

Human Cathepsin L1 (Sinobiological, 10486-H08H, no specific activity)

The assay buffer comprises 50 mM NaOAc, 100 mM NaCl, pH 3.5, 1 mM EDTA, and 5 mM DTT. rhCathepsin L1 was diluted to 40 μg/mL in assay buffer and incubated on ice for 15

minutes. The incubated rhCathepsin L was then diluted to 3.75 μg/mL in 50 μL of assay buffer, and the substrate was prepared to 40 μM in 0.5 μL of assay buffer. The protease and substrate were subsequently incubated at 37°C for 12 hours or longer. A portion of the mixture was then taken for LC-MS monitoring.

Human Cathepsin S (Sinobiological, 10487-H08H, >300 pmols/min/ug)

The assay buffer comprises 50 mM NaOAc, 250 mM NaCl, pH 4.5, 1 mM EDTA, and 5 mM DTT. RhCathepsin S was diluted to 15 μg/mL in the assay buffer to a final volume of 50 μL and incubated at room temperature for 2 hours. The substrate was diluted to 40 μM in the assay buffer to a final volume of 0.5 μL and mixed with the protease, followed by incubation at 37°C for 12 hours or longer. A portion of the mixture was then taken for LC-MS monitoring.

Human Cathepsin V (Sinobiological, 10093-H08H, >1000 pmols/min/ug)

The assay buffer comprises 25 mM NaOAc, 100 mM NaCl, pH 5.5, 1 mM EDTA, and 5 mM DTT. RhCathepsin V was diluted to 3.75 μg/mL in 50 μL of assay buffer, and the substrate was diluted to 40 μM in 0.5 μL of assay buffer. The protease and substrate were then incubated at 37°C for 12 hours or longer. A portion of the mixture was subsequently taken for LC-MS monitoring.

Human Cathepsin H (Sinobiological, 30063-H08H)

The activation buffer consists of 50 mM MES, 1 mM EDTA and 5 mM DTT, at pH 6.0. The assay buffer comprises 50 mM MES and 5 mM DTT, at pH 6.5. RhCathepsin L was diluted to 25 μg/mL in the activation buffer and incubated for 15 mins. RhCathepsin L and the activation buffer were added to rhCathepsin H to achieve final concentrations of 12.5 μg/mL rhCathepsin L and 50 μg/mL rhCathepsin H. The compounds were incubated at room temperature for 1 hour. The activated rhCathepsin H was then diluted to 0.5 μg/mL in the assay buffer, with a total volume of 50 μL, and the substrate was diluted to 40 μM. The protease and substrate were incubated at 37°C for 12 hours or longer. A portion of the mixture was subsequently taken for LC-MS monitoring.

Human Cathepsin Z/X (Sinobiological, 10159-H08H)

The activation buffer and the assay buffer comprises 25 mM NaOAc and 5 mM DTT, at pH 3.5. RhCathepsin Z was diluted to 100 μg/mL in the activation buffer and incubated for 5 mins. The activated rhCathepsin Z was then diluted to 2 μg/mL in the assay buffer, with a total volume of 50 μL, and the substrate was diluted to 40 μM. The protease and substrate were incubated at 37°C for 12 hours or longer. A portion of the mixture was subsequently taken for LC-MS monitoring.

Human carboxylesterases 1 (Sinobiological, 30105-H08H) and 2 (Sinobiological, 10380-H08H)

The assay buffer comprises 50 mM Tris, pH 7.5, and the reading buffer comprises 50 mM Tris, pH 8.0. Human carboxylesterases 1 was diluted to 0.1 μg/mL in the assay buffer, with a total volume of 50 μL, and the substrate was diluted to 40 μM. The mixture of 0.1 μg/mL human carboxylesterases 2 and 40 μM substrate incubated for 10 mins at room temperature, and then

add 1:1 volume of the reading buffer. The protease and substrate were incubated at 37°C for 12 hours or longer. A portion of the mixture was subsequently taken for LC-MS monitoring.

Pepsin (Sigma-Aldrich, P6887)

Pepsin was derived from porcine gastric mucosa, ⩾3,200 units/mg solid. To a solution of peptide (0.4 μL of a 4 mM stock solution in 10% DMSO/H2O, resulting in a final concentration of 0.04 mM) in 0.01 M HCl (pH 2.0, 39.2 μL), Pepsin (0.4 μL of a 0.75 mg/mL stock solution in PBS, resulting in a final concentration of 7.5 μg/mL, approximately 1 μmol: 500 U) was added subsequently. The protease and substrate were incubated at 37°C for 12 hours or longer. A portion of the mixture was subsequently taken for LC-MS monitoring.

Trypsin (Sigma-Aldrich, T4799)

Trypsin was derived from porcine pancreas, 1,000-2,000 BAEE units/mg solid. To a solution of peptide (0.4 μL of a 4 mM stock solution in 10% DMSO/H2O, resulting in a final concentration of 0.04 mM) in PBS buffer (pH 7.4, 39.2 μL), Trypsin (0.4 μL of a 0.08 mg/mL stock solution in PBS, resulting in a final concentration of 8 μg/mL, approximately 1 μmol: 200 U) was added subsequently. The protease and substrate were incubated at 37°C for 12 hours or longer. A portion of the mixture was subsequently taken for LC-MS monitoring.

Proteinase K (Meilunbiological, MA0006)

The assay buffer comprises 50 mM Tris-HCl and 10 mM $CaCl_2$, pH 8.0. Proteinase K was diluted to 50 μg/mL in the assay buffer, with a total volume of 50 μL, and the substrate was diluted to 40 μM. The protease and substrate were incubated at 37°C for 12 hours or longer. A portion of the mixture was subsequently taken for LC-MS monitoring.

Each protease was incubated with its peptide substrate under optimized conditions, and reaction progress was monitored by LC-MS. Cleavage rates toward different substrates were compared at identical time points. Using a 5% cleavage yield as the threshold, top-ranked sequences from each round were chosen for further multi-step screening, including site-directed mutagenesis and combinatorial comparison, until the sequence exhibiting the highest cleavage efficiency was identified.

The reported cleavage patterns and positive control results of some proteases are shown in Supplementary Data Fig. S7,8.

3. Protein sequences

Human pro-CATB

RSRPSFHPLSDELVNYVNKRNTTWQAGHNFYNVDMSYLKRLCGTFLGGPKPPQRVMFTEDLK
LPASFDAREQWPQCPTIKEIRDQGSCGSCWAFGAVEAISDRICIHTNAHVSVEVSAEDLLTC
CGSMCGDGCNGGYPAEAWNFWTRKGLVSGGLYESHVGCRPYSIPPCEHHVNGSRPPCTGEGD

TPKCSKICEPGYSPTYKQDKHYGYNSYSVSNSEKDIMAEIYKNGPVEGAFSVYSDFLLYKSG
VYQHVTGEMMGGHAIRILGWGVENGTPYWLVANSWNTDWGDNGFFKILRGQDHCGIESEVVA
GIPRTDQYWEKI

C29A mutant

RSRPSFHPLSDELVNYVNKRNTTWQAGHNFYNVDMSYLKRLCGTFLGGPKPPQRVMFTEDLK
LPASFDAREQWPQCPTIKEIRDQGSCGSAWAFGAVEAISDRICIHTNAHVSVEVSAEDLLTC
CGSMCGDGCNGGYPAEAWNFWTRKGLVSGGLYESHVGCRPYSIPPCEHHVNGSRPPCTGEGD
TPKCSKICEPGYSPTYKQDKHYGYNSYSVSNSEKDIMAEIYKNGPVEGAFSVYSDFLLYKSG
VYQHVTGEMMGGHAIRILGWGVENGTPYWLVANSWNTDWGDNGFFKILRGQDHCGIESEVVA
GIPRTDQYWEKI

S115A mutant

RPSFHPLSDELVNYVNKRNTTWQAGHNFYNVDMSYLKRLCGTFLGGPKPPQRVMFTEDLKLP
ASFDAREQWPQCPTIKEIRDQGSCGSCWAFGAVEAISDRICIHTNAHVSVEVSAEDLLTCCG
SMCGDGCNGGYPAEAWNFWTRKGLVSGGLYESHVGCRPYSIPPCEHHVNGARPPCTGEGDTP
KCSKICEPGYSPTYKQDKHYGYNSYSVSNSEKDIMAEIYKNGPVEGAFSVYSDFLLYKSGVY
QHVTGEMMGGHAIRILGWGVENGTPYWLVANSWNTDWGDNGFFKILRGQDHCGIESEVVAGI
PRTDQYWEKI

C29A-S115A mutant

RSRPSFHPLSDELVNYVNKRNTTWQAGHNFYNVDMSYLKRLCGTFLGGPKPPQRVMFTEDLK
LPASFDAREQWPQCPTIKEIRDQGSCGSAWAFGAVEAISDRICIHTNAHVSVEVSAEDLLTC
CGSMCGDGCNGGYPAEAWNFWTRKGLVSGGLYESHVGCRPYSIPPCEHHVNGARPPCTGEGD
TPKCSKICEPGYSPTYKQDKHYGYNSYSVSNSEKDIMAEIYKNGPVEGAFSVYSDFLLYKSG
VYQHVTGEMMGGHAIRILGWGVENGTPYWLVANSWNTDWGDNGFFKILRGQDHCGIESEVVA
GIPRTDQYWEKI

Mature hCATB

LPASFDAREQWPQCPTIKEIRDQGSCGSCWAFGAVEAISDRICIHTNAHVSVEVSAEDLLTC
CGSMCGDGCNGGYPAEAWNFWTRKGLVSGGLYESHVGCRPYSIPPCEHHVNGSRPPCTGEGD
TPKCSKICEPGYSPTYKQDKHYGYNSYSVSNSEKDIMAEIYKNGPVEGAFSVYSDFLLYKSG
VYQHVTGEMMGGHAIRILGWGVENGTPYWLVANSWNTDWGDNGFFKILRGQDHCGIESEVVA
GIPRTDQYWEKI

Mature hCATB-S115A

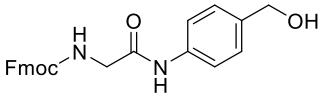
LPASFDAREQWPQCPTIKEIRDQGSCGSCWAFGAVEAISDRICIHTNAHVSVEVSAEDLLTC
CGSMCGDGCNGGYPAEAWNFWTRKGLVSGGLYESHVGCRPYSIPPCEHHVNGARPPCTGEGD
TPKCSKICEPGYSPTYKQDKHYGYNSYSVSNSEKDIMAEIYKNGPVEGAFSVYSDFLLYKSG
VYQHVTGEMMGGHAIRILGWGVENGTPYWLVANSWNTDWGDNGFFKILRGQDHCGIESEVVA
GIPRTDQYWEKI

4. Chemical Synthesis

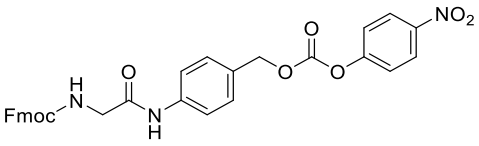
Synthesis of Ac-rkyyG-Cl for co-crystallization

Standard Fmoc solid-phase peptide synthesis methods were employed on 2-chlorotrityl chloride resin (0.5 mmol/g, 500 mg) to synthesize Ac-r(Pbf)k(Boc)y(tBu)y(tBu)-OH. The crude peptide yield was 700 mg. Ac-r(Pbf)k(Boc)y(tBu)y(tBu)-OH (350 mg, 0.308 mmol), 1-Amino-3-chloropropan-2-one (32 mg, 0.308 mmol), HATU (117 mg, 0.308 mmol), and pyridine (73 mg, 0.308 mmol) were dissolved in DMF (5 mL) and stirred at room temperature for 2 hours. Upon completion of the reaction, the mixture was diluted with deionized water and extracted with EtOAc (3×). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuum. The crude product was purified by flash column chromatography (5% CH_2Cl_2 in MeOH) to yield the product (60 mg, 16%) as a white solid. This product was then dissolved in 3 mL DCM, and 3 mL TFA was added. The reaction proceeded for 20 minutes at room temperature. The resulting crude product was purified by HPLC using an Agilent C18 column and lyophilized to obtain the final product, Ac-rkyyG-Cl, as a white powder (20 mg, 54%). HRMS calculated for $C_{35}H_{50}ClN_9O_8$ $[M+H]^+$ 760.3549 Da, observed at 760.3505 Da (Supplementary Data S3a). Using the same synthetic approach, the product Ac-YYG-Cl was obtained. HRMS calculated for $C_{23}H_{26}ClN_3O_6$ $[M+H]^+$ 475.1510 Da, observed at 476.1571 Da (Supplementary Data S3c).

Synthesis of DBCO-linker-MMAE for ADC conjugation

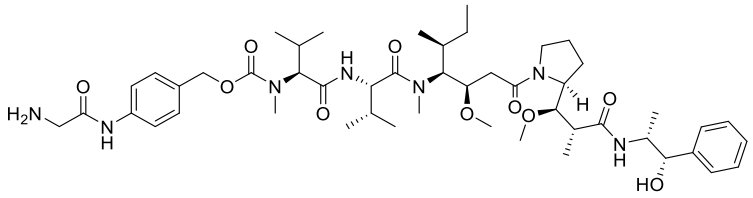


Fmoc-G-PAB-OH (1a): Fmoc-Gly-OH (892 mg, 3 mmol) and TSTU (1084 mg, 3.6 mmol) were dissolved in DMF (10 mL). N, N-Diisopropylethylamine (775 mg, 6 mmol) was added to the mixture, which was then stirred at room temperature for 3 h. 4-Aminobenzyl alcohol (369 mg, 3 mmol) was subsequently added to the reaction mixture, and the reaction was allowed to proceed for an additional 2 h. Upon completion of the reaction, the mixture was diluted with deionized water and extracted with EtOAc (3×). The combined organic layers were washed with brine, dried over $MgSO_4$, and concentrated in vacuo. The crude product was purified by flash column chromatography (5% CH_2Cl_2 in MeOH) to afford the product **1a** (400 mg, 33%) as a white solid. HRMS calculated for $C_{24}H_{22}N_2O_4$ $[M+H]^+$ 403.1658 Da, observed 403.1700 Da.

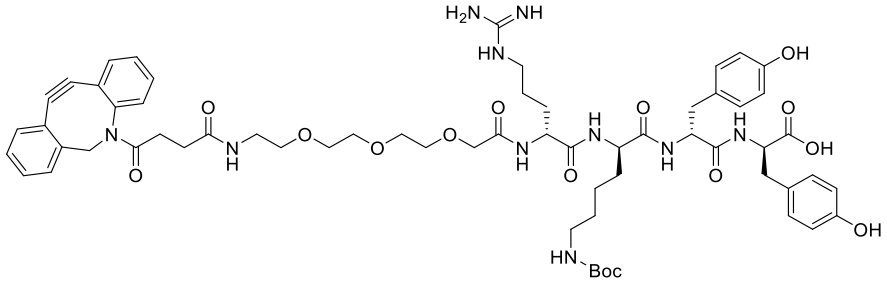


Fmoc-G-PAB-PNP (1b): 1a (80 mg, 0.2 mmol) and bis(4-nitrophenyl) carbonate (308 mg, 1.0 mmol) were dissolved in DCM (3 mL). 4-Dimethylaminopyridine (50 mg, 0.4 mmol) was added to the mixture, which was then stirred at room temperature for 2 h. The reaction mixture

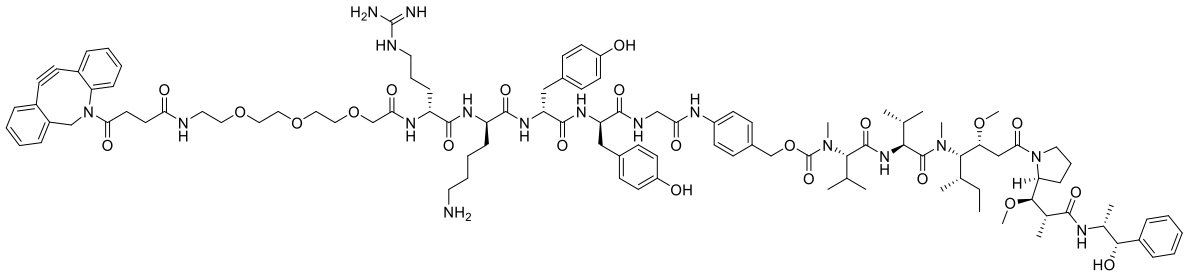
was filtered to afford the product **1b** (90 mg, 80%). HRMS calculated for $C_{31}H_{25}N_3O_8$ $[M+H]^+$ 568.1720 Da, observed: 568.1691 Da.



G-PAB-MMAE (1c): 1b (57 mg, 0.1 mmol), MMAE (110 mg, 0.15 mmol), and HOAt (30 mg, 0.2 mmol) were dissolved in DCM (5 mL). N, N-Diisopropylethylamine (0.5 mL, 0.5 mmol) was added to the mixture, which was then stirred at room temperature for 30 minutes. Piperidine (1 mL) was subsequently added to the reaction mixture, and the reaction proceeded for 5 minutes. The reaction mixture was concentrated under vacuum, and the resulting crude product was purified by HPLC using an Agilent C18 column. The purified product was lyophilized to yield **1c** as a white powder (65 mg, 70%). HRMS calculated for $C_{49}H_{77}N_7O_{10}$ $[M+H]^+$ 924.5810 Da, observed 924.5841 Da.

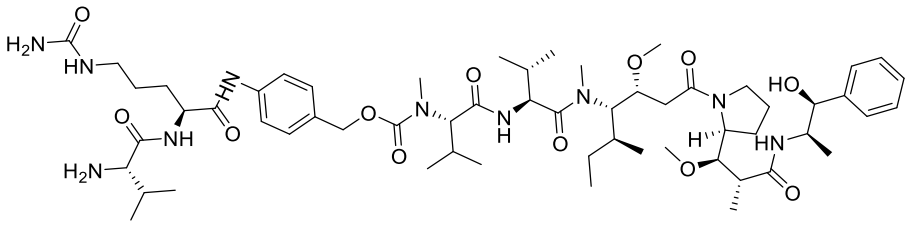


DBCO-PEG3-rk(Boc)yy-OH (1d): Employed standard Fmoc solid-phase peptide synthesis methods on 2-chlorotrityl chloride resin (0.5 mmol/g, 500 mg) to synthesize **1d**. D-Arg-OH and D-Tyr-OH were used without side-chain protection. Couplings were conducted using a 5-fold molar excess of each amino acid, DIC, and Oxyma. Final deprotection and cleavage were performed with 1% TFA in DCM for 1 hour. TEA was added to neutralize the solution before concentration. The crude product was purified by HPLC using an Agilent C18 column and lyophilized to yield a white powder (50 mg, 17%). HRMS calculated for $C_{62}H_{80}N_{10}O_{15}$ $[M+H]^+$ 1205.5882 Da; observed 1205.5760 Da.

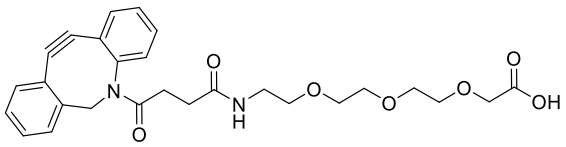


DBCO-PEG3-rkyyG-PAB-MMAE (1e): 1d (40 mg, 0.033 mmol), **1c** (30 mg, 0.032 mmol), DIC (4.2 mg, 0.033 mmol), and Oxyma (4.7 mg, 0.033 mmol) were dissolved in DMF (5 mL) and stirred at room temperature for 2 h. The resulting crude product was purified by HPLC using an Agilent C18 column and lyophilized to yield a protected product as a white powder

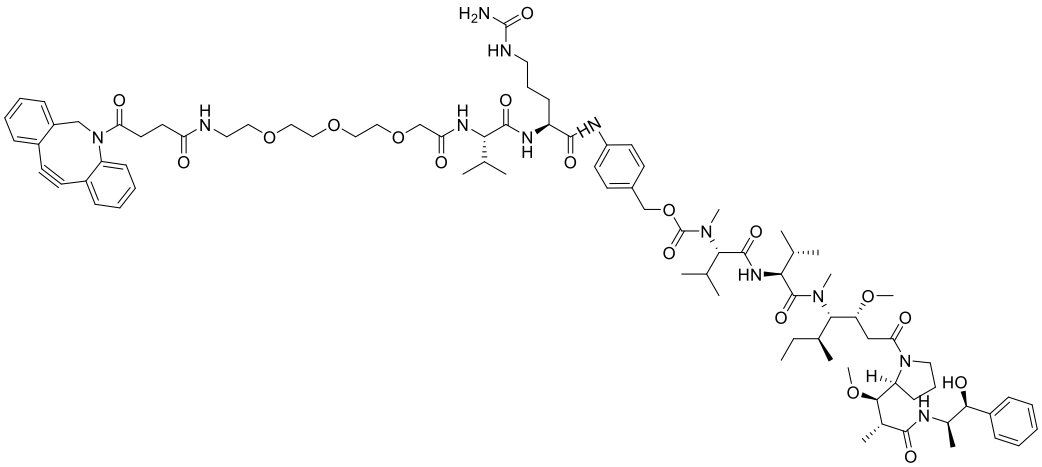
(15 mg, 21%). The product was then dissolved in 1 mL DCM, and 0.25 mL TFA was added. The mixture was stirred at room temperature for 30 minutes. The resulting crude product was purified by HPLC using an Agilent C18 column and lyophilized to yield **1e** as a white powder (2.7 mg, 18%). HRMS calculated for $C_{106}H_{147}N_{17}O_{22}$ $[M+H]^+$ 2011.0984 Da, observed 2011.0697 Da (Supplementary Data S3e).



Val-Cit-PAB-MMAE (1f): Fmoc-Val-Cit-PAB-PNP (77 mg, 0.1 mmol, CAS 863971-53-3), MMAE (72 mg, 0.1 mmol), and HOAt (30 mg, 0.2 mmol) were dissolved in DCM (5 mL). N, N-Diisopropylethylamine (0.5 mL, 0.5 mmol) was added to the mixture, which was then stirred at room temperature for 30 minutes. Piperidine (1 mL) was subsequently added to the reaction mixture, and the reaction proceeded for 5 minutes. The reaction mixture was concentrated under vacuum. The resulting crude product was purified by HPLC using an Agilent C18 column and lyophilized to yield **1f** as a white powder (55 mg, 49%). HRMS calculated for $C_{58}H_{94}N_{10}O_{12}$ $[M+H]^+$ 1123.7131, observed 1123.7028.

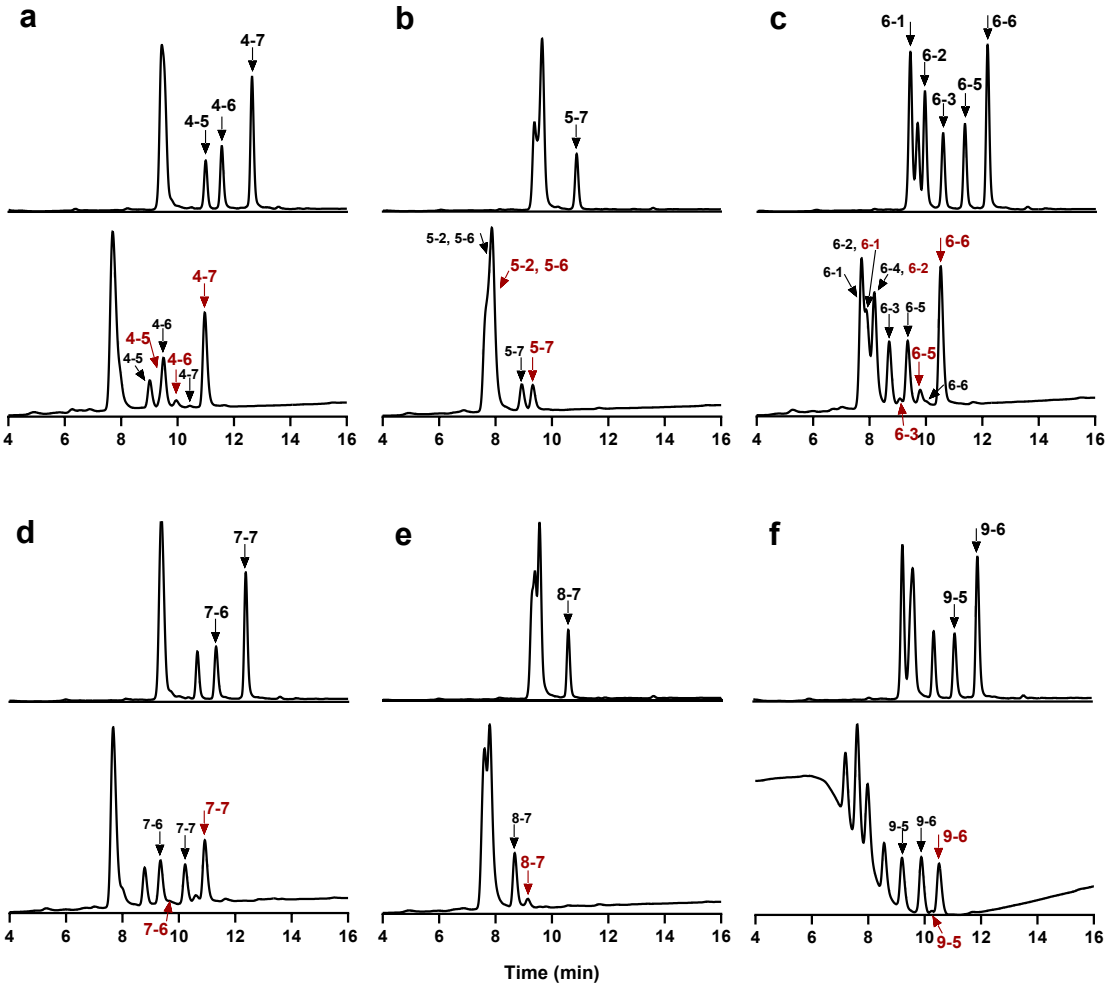


DBCO-PEG3-OH (1g): Utilized standard Fmoc solid-phase peptide synthesis methods on 2-chlorotrityl chloride resin (0.5 mmol/g, 500 mg) to synthesize DBCO-PEG3-OH. Couplings were conducted using a 5-fold molar excess of each amino acid, DIC, and Oxyma. For the final peptide deprotection and cleavage, a 1% TFA in DCM solution was used for 1 hour. Triethylamine (TEA) was subsequently added to neutralize the solution prior to concentration. The crude product was directly utilized for the subsequent reaction without purification.

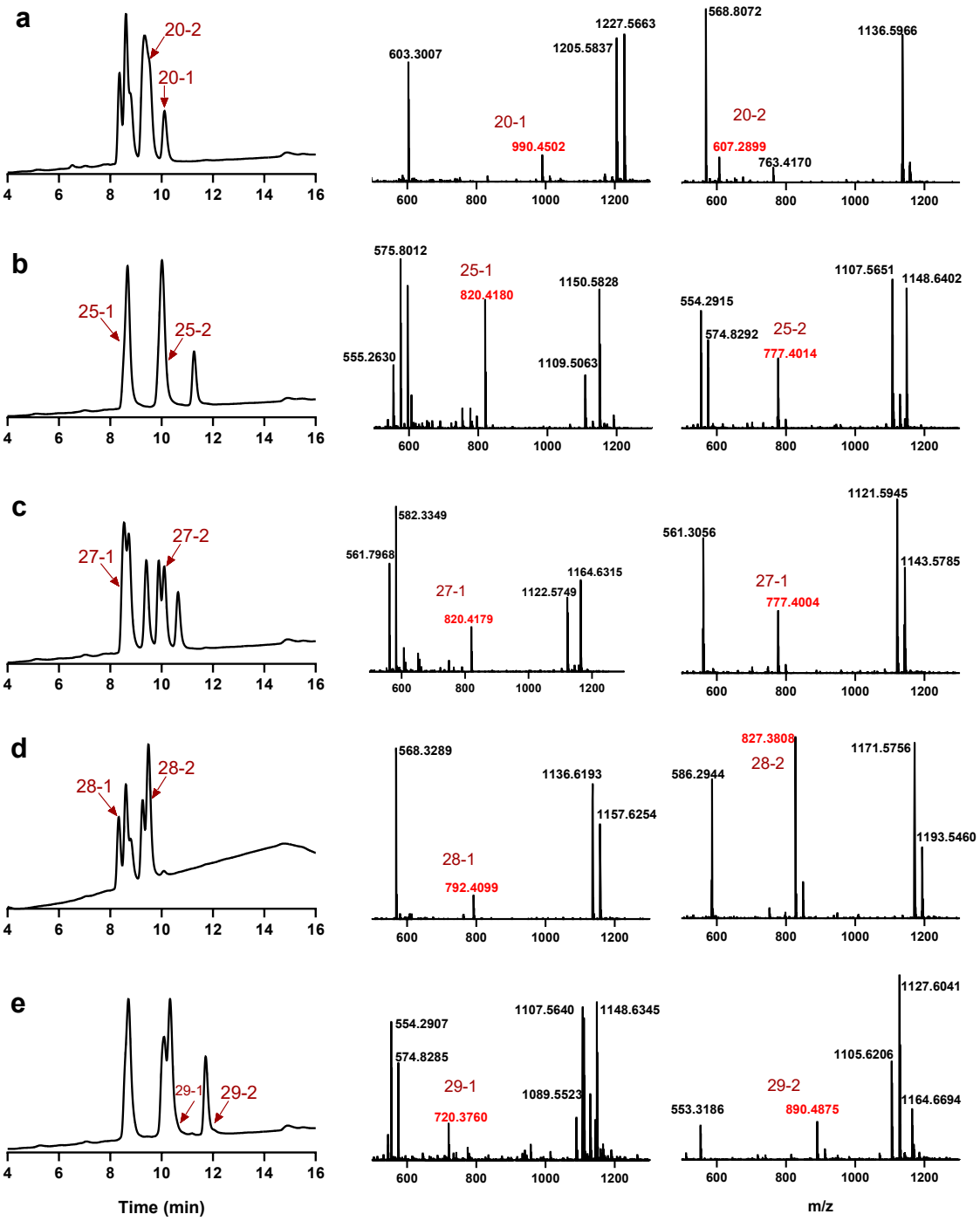


DBCO-PEG3-Val-Cit-PAB-MMAE (1h): 1g (25 mg, 0.05 mmol), **1f** (56 mg, 0.05 mmol), HATU (19 mg, 0.05 mmol), and DIEA (20 mg, 0.15 mmol) were dissolved in DCM (5 mL) and stirred at room temperature for 1 hour. The resulting crude product was purified by HPLC using an Agilent C18 column and lyophilized to yield **1h** as a white powder (5 mg, 6%). HRMS calculated for $C_{85}H_{122}N_{12}O_{18}$ $[M+H]^+$ 1599.9078, observed: 1599.9008 (Supplementary Data S3f).

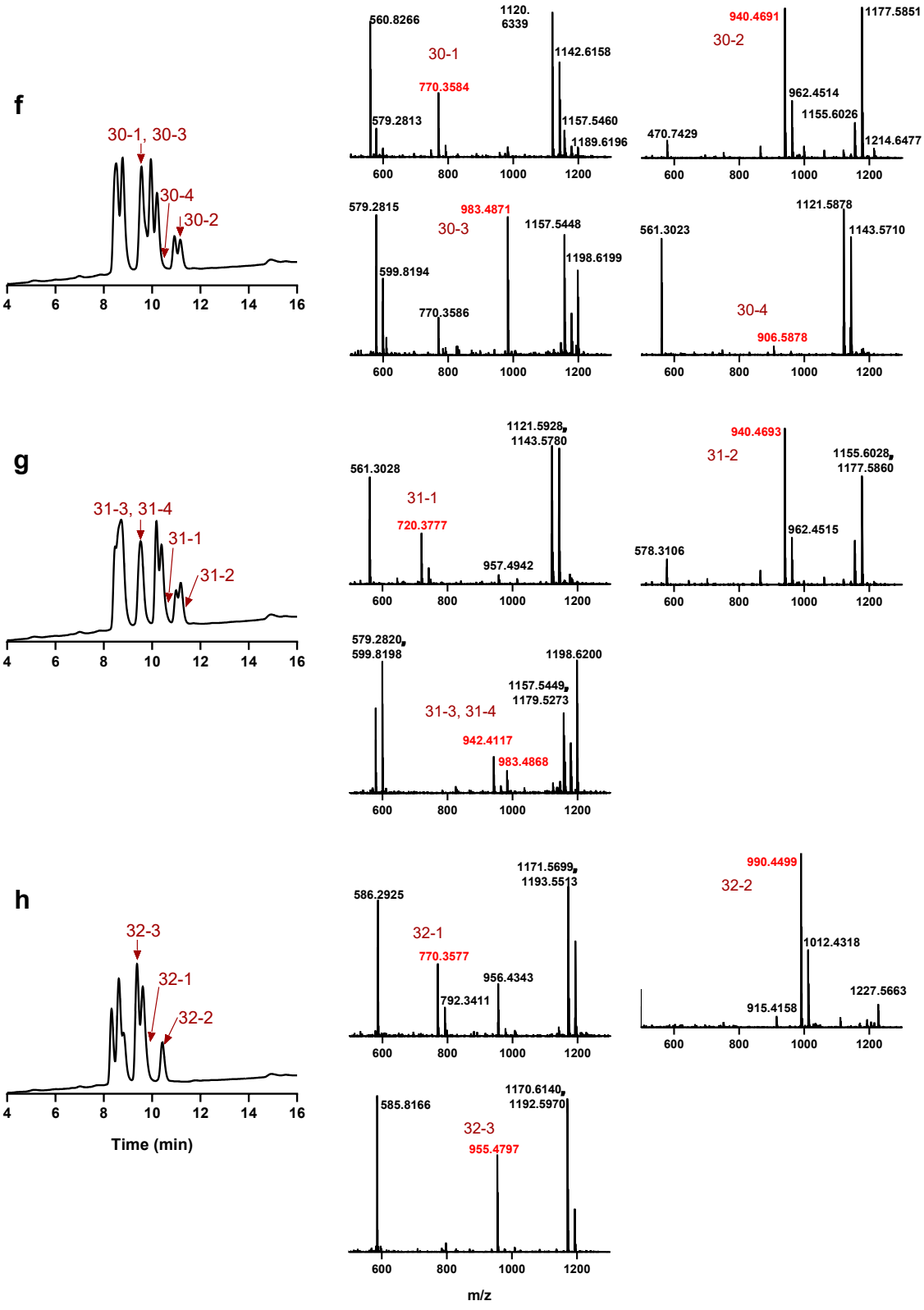
Supplementary Data and Tables



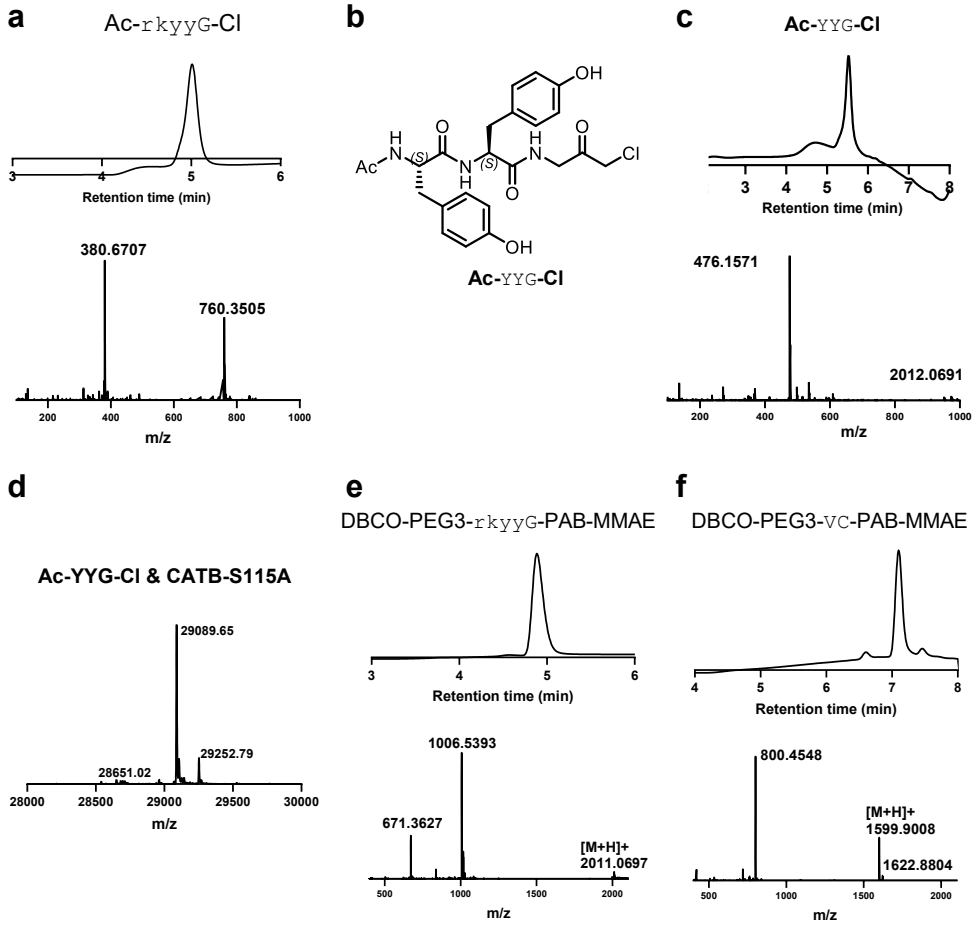
Supplementary Data S1. LC-MS analysis of papain enzymatic reaction on peptide mixture 4-9 in Supplementary Table S2. The 214 nm absorption spectra of the samples are shown. The upper panels in each group depict the mixed peptides before adding the enzyme, while the lower panels illustrate the mixed peptides treated with papain (37°C for 36 hours). Black arrows indicate the raw materials, whereas red arrows denote the cleaved products. The HPLC gradient for the initial mixture peptide analysis was 5-46% *solvent B* over 17 minutes, followed by 46-61% solvent B over 1 minute. The HPLC gradient for the product analysis after enzyme treatment was 5-46% *solvent B* over 13 minutes, followed by 46-61% *solvent B* over 1 minute.



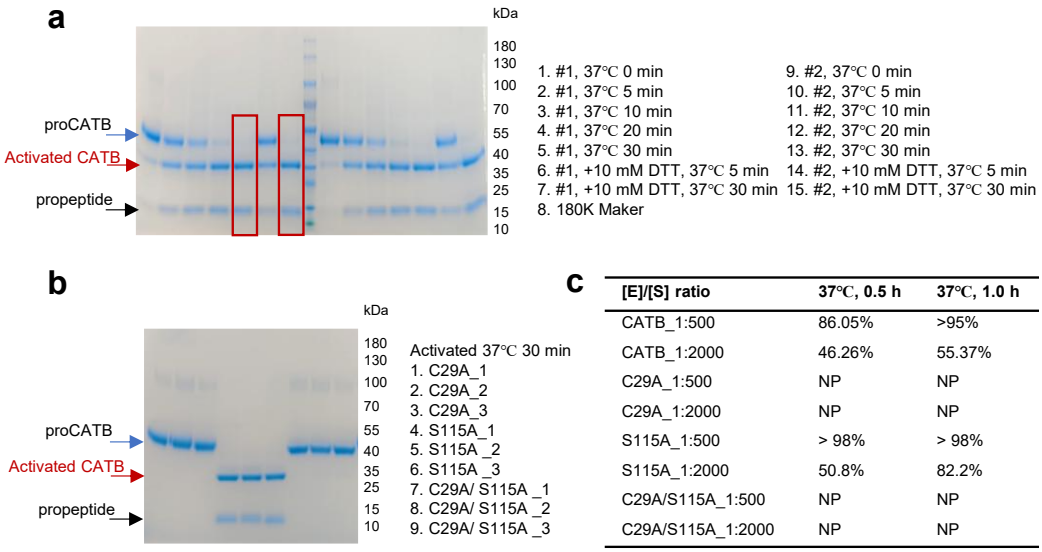
Supplementary Data S2. (Continued)



Supplementary Data S2. LC-MS analysis of papain cleavage of sequences listed in Supplementary Table S6. The 214 nm absorption spectra of samples are shown in the left panels, and the corresponding MS results are displayed in the right panels. Red labels indicate the cleaved products. The HPLC gradient for the product analysis after enzyme treatment was 5-46% solvent B over 13 minutes, followed by 46-61% solvent B over 1 minute.



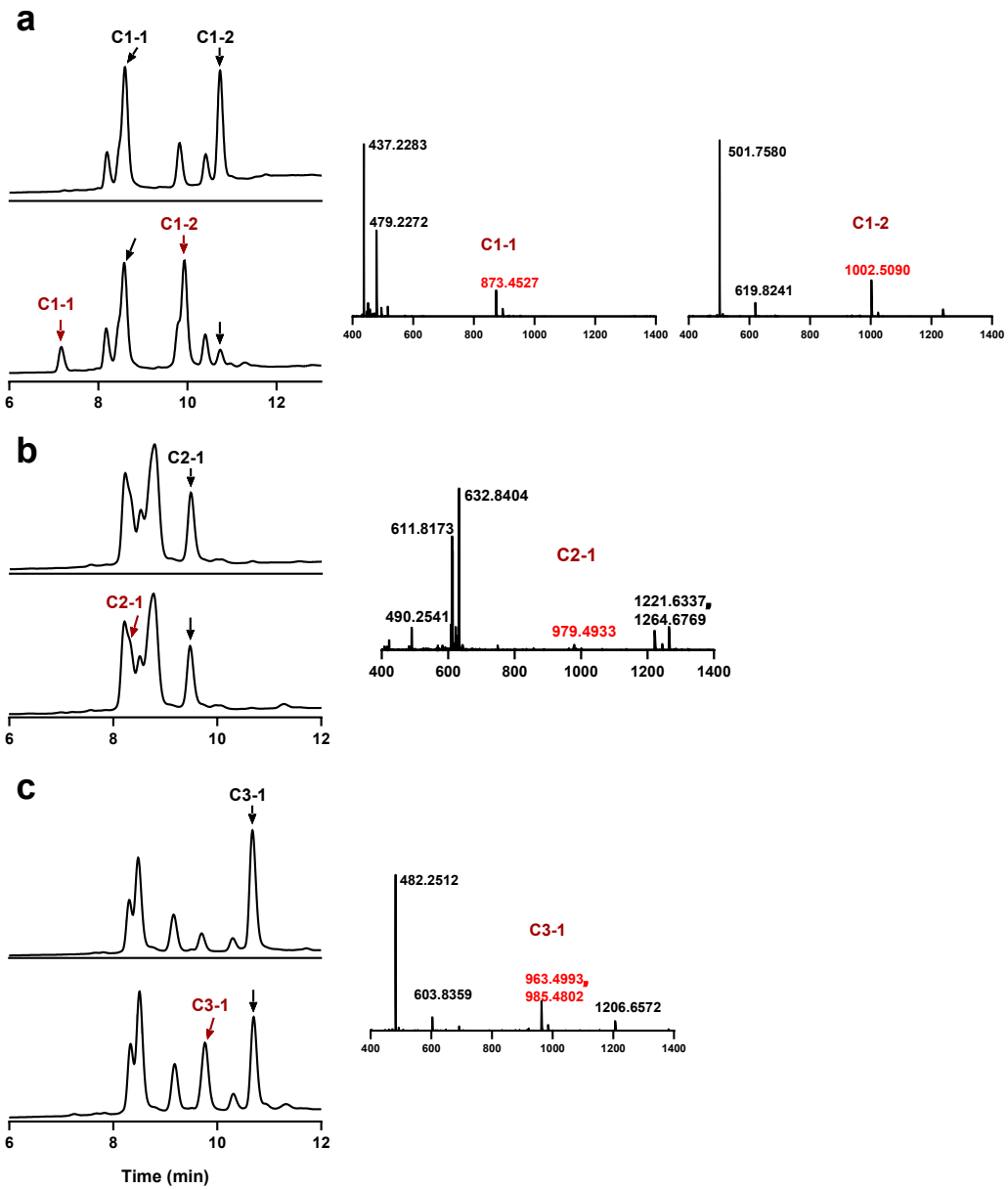
Supplementary Data S3. Chemical characterization of synthetic peptides. a,c,e,f, HPLC and mass spectrometry to determine the purity of the synthesized peptide Ac-rkyyG-Cl (**a**), Ac-YYG-Cl (**c**), DBCO-PEG3-rkyyG-PAB-MMAE (**e**), and DBCO-PEG3-VC-PAB-MMAE (**f**). The top panel is the HPLC structure, and the bottom panel is the MS results. **b**, the structure of Ac-YYG-Cl, covalently targeting the catalytic Cys29 of hCATB. **d**, Deconvoluted LC-MS confirms that Ac-YYG-Cl covalently binds to hCATB-S115A.



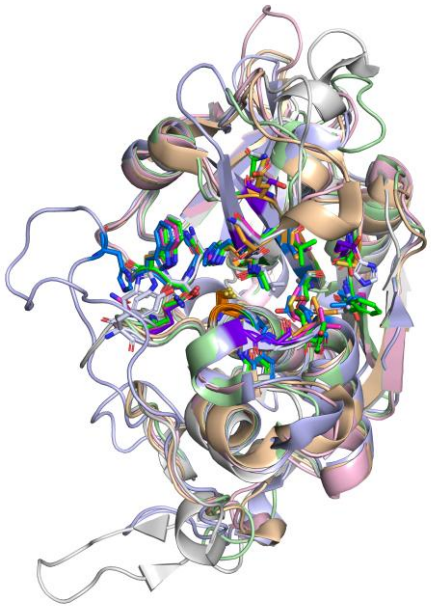
c

[E]/[S] ratio	37℃, 0.5 h	37℃, 1.0 h
CATB_1:500	86.05%	>95%
CATB_1:2000	46.26%	55.37%
C29A_1:500	NP	NP
C29A_1:2000	NP	NP
S115A_1:500	> 98%	> 98%
S115A_1:2000	50.8%	82.2%
C29A/S115A_1:500	NP	NP
C29A/S115A_1:2000	NP	NP

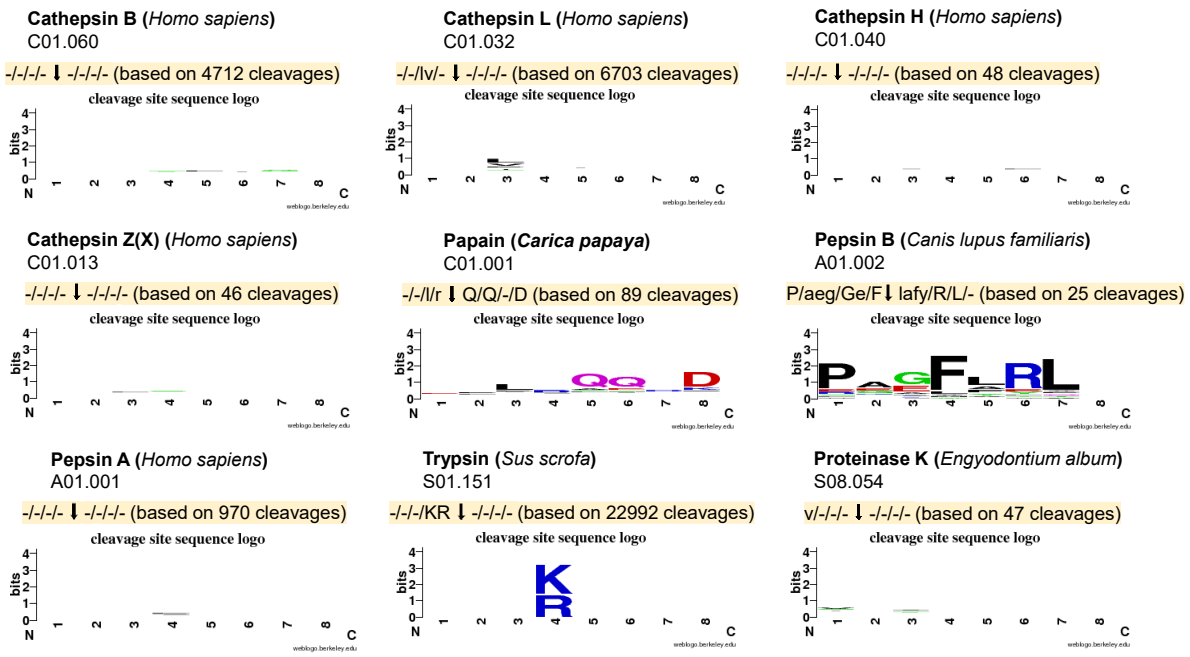
Supplementary Data S4. Activation of hCATB and mutants. a, The activation of hCATB in the MES buffer at 37°C was confirmed through SDS-PAGE. **b,** SDS-PAGE analysis of active hCATB mutants indicates that hCATB-S115A is successfully activated. **c,** Enzymatic activity assays of hCATB mutants demonstrate that hCATB-S115A retains activity comparable to that of wild-type hCATB, whereas the dead mutant C29A exhibits a loss of activity.



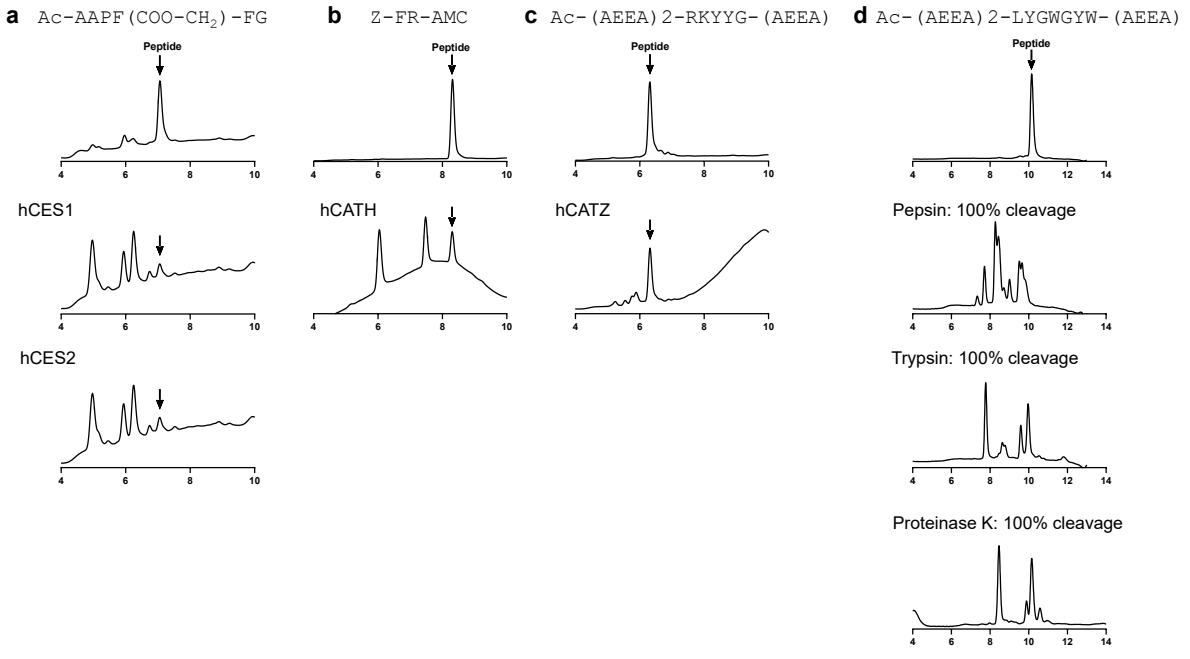
Supplementary Data S5. LC-MS analysis of mCES1c cleavage of D-peptides in Supplementary Table S30. The 214 nm absorption spectra of the samples are displayed in the left panels, with the corresponding MS results presented in the right panels. The upper panels of each group depict the initial mixed peptides, whereas the lower panels show the mixed D-peptides treated with mCES1c, all incubated at 37°C for 24 hours. Black arrows and labels indicate the raw materials, whereas red arrows denote the cleaved products. The HPLC gradient for analysis was 5-46% solvent B over 13 minutes, followed by 46-61% solvent B over 1 minute.



Supplementary Data S6. Structure alignment of papain, hCATB, hCATH, hCATL and hCATZ. The schematic representations illustrate the structures of papain (1CVZ, green), hCATB (6AY2, blue), hCATH (6CZS, orange), hCATL (2XU3, pink), and hCATZ (1EF7, gray). Key residues of each protease are shown as dark-colored sticks: papain (Gln19, Cys25, Trp26, Tyr67, Pro68, Ser131, Val133, Asp157, His159, Ala160, Asn175, Ser176, Trp177, Ser205, Phe207); hCATB (Gln23, Cys29, Trp30, Pro76, His110, Glu171, Ala173, Gly198, His199, Ala200, Asn129, Ser220, Trp221, Glu245, Val247); hCATH (Gln135, Cys141, Leu185, Pro186, Ser250, Ala252, Asn280, His281, Ala282, Asn301, Ser302, Trp303, Cys327, Ser329); hCATL (Asn18, Gln19, Cys25, Trp16, Leu69, Met70, Ser133, Ala135, Asp162, His163, Gly164, Ser188, Trp189, Ala214, Ser216); and hCATZ (Gln22, His23, Cys31, Trp32, Asp76, Ser152, Asn179, His180, Val181, Asn200, Ser201, Trp202, His234, Thr236). Residues of hCATB that interact with the D-peptide substrate (-rkyyG-) are depicted as purple sticks.

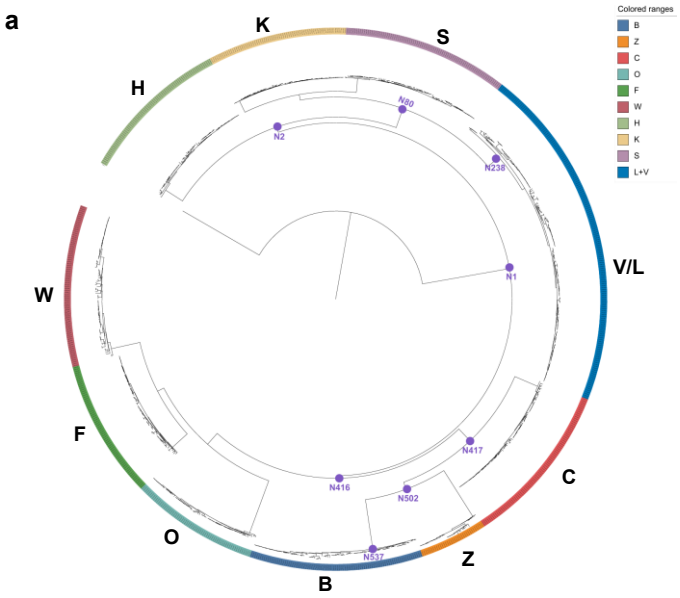


Supplementary Data S7. Cleavage patterns of proteases toward L-amino acid substrates. Data were obtained from MEROPS - the Peptidase Database[52]. No corresponding cleavage profiles are available for human or mouse carboxylesterases 1 and 2. Yellow-marked areas: Uppercase letters indicate strong preference, lowercase letters indicate low preference. Except for pepsin, which is derived from porcine gastric mucosa during experiments, the sources of all other proteases align with the species origins depicted in the figure.



Supplementary Data S8. Evaluation of protease activity under reaction conditions using known L-peptide substrates. Reaction conditions for each protease are detailed in the **Methods** section. In the HPLC chromatograms, black arrows indicate the remaining substrate (substrate sequence shown above).

a



b

Enzyme concentration	Reaction time (min)	Yield CATB	Yield N537
2 μg/mL	5	42.9%	**42.0%**
	15	82.1%	**83.3%**
	30	>95%	**>95%**
	60	100%	**100%**
0.5 μg/mL	5	13.7%	**14.3%**
	15	24.2%	**25.6%**
	30	38.4%	**43.7%**
	60	64.5%	**69.8%**

c

Enzyme concentration	Reaction time (hour)	Yield CATL	Yield N238
20 μg/mL	4	27.8%	**2%**
	8	41.3%	**3%**
	12	47.4%	**5%**
	16	51.9%	**9%**
	24	61.6%	**10%**

Supplementary Data S9. Phylogenetic analysis of ten cathepsins. a, Tree constructed from amino acid sequences of ten cathepsins. Ancestral nodes of cathepsin B and L are purple; cathepsin V and L (high sequence similarity) are highlighted in dark blue. **b-c,** The ancestral sequence of cathepsin B (N537; **b**) and cathepsin L (N238; **c**) were successfully expressed, and their heterochiral cleavage activity towards the corresponding optimal D-peptide substrates were validated.

Supplementary Table S1. Information of D-amino acids and Gly. The enantiomeric purity of certain amino acids is provided by GL Biochem. The molar ratio of amino acids used in the mixture is shown.

Amino acid	Enantiomeric purity (%)	Molar ratio
a	99.86	0.75
d	> 99.9	1.20
e	99.84	1.00
f	> 99.9	0.60
G	100	0.70
h	> 99.9	0.78
i	99.37	2.29
k	99.94	1.00
l	> 99.9	0.77
m	99.52	0.71
n	> 99.9	1.20
p	99.89	0.86
q	> 99.9	1.00
r	99.74	1.10
s	99.92	0.71
t	99.98	0.98
v	99.9	1.80
w	99.86	0.70
y	> 99.9	0.71

Supplementary Table S2. Sequences included in Library #1.

	Sequence		Sequence
1	Ac-wsp**GGG**$\mathbf{x_1}$as	**7**	Ac-wsp**G**$\mathbf{x_1}$**GG**as
2	Ac-wsp**GGG**$\mathbf{x_2}$as	**8**	Ac-wsp**G**$\mathbf{x_2}$**GG**as
3	Ac-wsp**GGG**$\mathbf{x_3}$as	**9**	Ac-wsp**G**$\mathbf{x_3}$**GG**as
4	Ac-wsp**GG**$\mathbf{x_1}$**G**as	**10**	Ac-wsp$\mathbf{x_1}$**GGG**as
5	Ac-wsp**GG**$\mathbf{x_2}$**G**as	**11**	Ac-wsp$\mathbf{x_2}$**GGG**as
6	Ac-wsp**GG**$\mathbf{x_3}$**G**as	**12**	Ac-wsp$\mathbf{x_3}$**GGG**as

$x_1 = k + s + d + G + m + l + w$

$x_2 = n + h + e + a + t + y$

$x_3 = q + r + v + p + i + f$

$x = x_1 + x_2 + x_3$

Ac-wsp- and -as- were appended to the N-terminus and C-terminus of the target screening sequences to ensure successful HPLC analysis of the peptide cleavage. These appended D-peptides also ensure that any observed proteolytic activity results from the recognition of D-peptide substrates. This principle was applied to subsequent experiments. All samples were incubated at 37°C for 36 hours.

Supplementary Table S3. All tested sequences in Library #1 by papain.

	Sequence		Sequence		Sequence
1-1	Ac-wsp**GGGk**as	**2-1**	Ac-wsp**GGGn**as	**3-1**	Ac-wsp**GGGq**as
1-2	Ac-wsp**GGGs**as	**2-2**	Ac-wsp**GGGh**as	**3-2**	Ac-wsp**GGGr**as
1-3	Ac-wsp**GGGd**as	**2-3**	Ac-wsp**GGGc**as	**3-3**	Ac-wsp**GGGv**as
1-4	Ac-wsp**GGGG**as	**2-4**	Ac-wsp**GGGe**as	**3-4**	Ac-wsp**GGGp**as
1-5	Ac-wsp**GGGm**as	**2-5**	Ac-wsp**GGGa**as	**3-5**	Ac-wsp**GGGi**as
1-6	Ac-wsp**GGGl**as	**2-6**	Ac-wsp**GGGt**as	**3-6**	Ac-wsp**GGGf**as
1-7	Ac-wsp**GGGw**as	**2-7**	Ac-wsp**GGGy**as		
4-1	Ac-wsp**GGkG**as	**5-1**	Ac-wsp**GGnG**as	**6-1**	Ac-wsp**GGqG**/as
4-2	Ac-wsp**GGsG**as	**5-2**	Ac-wsp**GGhG**/as	**6-2**	Ac-wsp**GGrG**/as
4-3	Ac-wsp**GGdG**as	**5-3**	Ac-wsp**GGcG**as	**6-3**	Ac-wsp**GGvG**/as
4-4	Ac-wsp**GGGG**as	**5-4**	Ac-wsp**GGeG**as	**6-4**	Ac-wsp**GGpG**as
4-5	Ac-wsp**GGmG**/as	**5-5**	Ac-wsp**GGaG**as	**6-5**	Ac-wsp**GGiG**/as
4-6	Ac-wsp**GGlG**/as	**5-6**	Ac-wsp**GGtG**/as	**6-6**	Ac-wsp**GGfG**/as
4-7	Ac-wsp**GGwG**/as	**5-7**	Ac-wsp**GGyG**/as		
7-1	Ac-wsp**GkG/G**as	**8-1**	Ac-wsp**GnGG**as	**9-1**	Ac-wsp**GqGG**as
7-2	Ac-wsp**GsGG**as	**8-2**	Ac-wsp**GhGG**as	**9-2**	Ac-wsp**GrG/G**as
7-3	Ac-wsp**GdGG**as	**8-3**	Ac-wsp**GcGG**as	**9-3**	Ac-wsp**GvG/G**as
7-4	Ac-wsp**GGGG**as	**8-4**	Ac-wsp**GeGG**as	**9-4**	Ac-wsp**GpGG**as
7-5	Ac-wsp**GmG/G**as	**8-5**	Ac-wsp**GaGG**as	**9-5**	Ac-wsp**GiG/G**as
7-6	Ac-wsp**GlG/G**as	**8-6**	Ac-wsp**GtGG**as	**9-6**	Ac-wsp**GfG/G**as
7-7	Ac-wsp**GwG/G**as	**8-7**	Ac-wsp**GyG/G**as		
10-1	Ac-wsp**kGGG**as	**11-1**	Ac-wsp**nGGG**as	**12-1**	Ac-wsp**qGGG**as
10-2	Ac-wsp**sGGG**as	**11-2**	Ac-wsp**hGGG**as	**12-2**	Ac-wsp**rGGG**as
10-3	Ac-wsp**dGGG**as	**11-3**	Ac-wsp**cGGG**as	**12-3**	Ac-wsp**vGGG**as
10-4	Ac-wsp**GGGG**as	**11-4**	Ac-wsp**eGGG**as	**12-4**	Ac-wsp**pGGG**as
10-5	Ac-wsp**mGGG**as	**11-5**	Ac-wsp**aGGG**as	**12-5**	Ac-wsp**iGGG**as
10-6	Ac-wsp**lGGG/**as	**11-6**	Ac-wsp**tGGG**as	**12-6**	Ac-wsp**fGGG**as
10-7	Ac-wsp**wGGG/**as	**11-7**	Ac-wsp**yGGG**as		

Slash denotes the cleavage site by papain. The presumable recognized D-amino acids in different peptides are highlighted in red. Sequences highlighted in orange exhibited cleavage yields exceeding 80% in mixed peptides after 36 hours at 37°C with papain. The concentration of papain was 235 nM, and of substrates was 40 μM.

Supplementary Table S4. Preferred sequences recognized by papain based on Library #1.

	Sequence	Yield (36 h)
4-7	Ac-wsp**GGwG/**as	> 90%
6-6	Ac-wsp**GGfG/**as	> 90%
7-7	Ac-wsp**GwG/G**as	> 90%
5-7	Ac-wsp**GGyG/**as	> 40%
8-7	Ac-wsp**GfG/G**as	> 40%

Slash denotes the cleavage site by papain.

Supplementary Table S5. Sequences included in Library #2.

	Sequence		Sequence
13	Ac-AEEA-wsa**GGz$_1$z$_1$**a-AEEA	**25**	Ac-AEEA-wsa**Gz$_1$Gz$_1$**a-AEEA
14	Ac-AEEA-wsa**GGz$_2$z$_1$**a-AEEA	**26**	Ac-AEEA-wsa**Gz$_2$Gz$_1$**a-AEEA
15	Ac-AEEA-wsa**GGz$_1$z$_2$**a-AEEA	**27**	Ac-AEEA-wsa**Gz$_1$Gz$_2$**a-AEEA
16	Ac-AEEA-wsa**GGz$_2$z$_2$**a-AEEA	**28**	Ac-AEEA-wsa**Gz$_2$Gz$_2$**a-AEEA
17	Ac-AEEA-wsa**Gz$_1$z$_1$G**a-AEEA	**29**	Ac-AEEA-wsa**z$_1$Gz$_1$G**a-AEEA
18	Ac-AEEA-wsa**Gz$_2$z$_1$G**a-AEEA	**30**	Ac-AEEA-wsa**z$_2$Gz$_1$G**a-AEEA
19	Ac-AEEA-wsa**Gz$_1$z$_2$G**a-AEEA	**31**	Ac-AEEA-wsa**z$_1$Gz$_2$G**a-AEEA
20	Ac-AEEA-wsa**Gz$_2$z$_2$G**a-AEEA	**32**	Ac-AEEA-wsa**z$_2$Gz$_2$G**a-AEEA
21	Ac-AEEA-wsa**z$_1$z$_1$GG**a-AEEA	**33**	Ac-AEEA-wsa**z$_1$GGz$_1$**a-AEEA
22	Ac-AEEA-wsa**z$_2$z$_1$GG**a-AEEA	**34**	Ac-AEEA-wsa**z$_2$GGz$_1$**a-AEEA
23	Ac-AEEA-wsa**z$_1$z$_2$GG**a-AEEA	**35**	Ac-AEEA-wsa**z$_1$GGz$_2$**a-AEEA
24	Ac-AEEA-wsa**z$_2$z$_2$GG**a-AEEA	**36**	Ac-AEEA-wsa**z$_2$GGz$_2$**a-AEEA

z_1 = r + d + l

z_2 = k + e + y

$z = z_1 + z_2$

Ac-AEEA-wsa- and -a-AEEA- were appended to the N-terminus and C-terminus of the target screening sequences to ensure successful HPLC analysis of the peptide cleavage. All samples were incubated at 37°C for 36 hours.

Supplementary Table S6. Observed sequences recognized by papain based on Library #2.

	Sequence	Main cleaved fragment MW (kDa)	Intact peptide MW (kDa)
20-1	Ac-AEEA-wsa**GyyG/**a-AEEA	990.40	1205.52
20-2	Ac-AEEA-wsa**G/yyG**a-AEEA	607.25	1205.52
25-1	Ac-AEEA-wsa**GrG/z$_1$**a-AEEA	820.37	1191.59, 1150.51, 1148.57
25-2	Ac-AEEA-wsa**GlG/z$_1$**a-AEEA	777.35	1148.57, 1107.50, 1105.55
27-1	Ac-AEEA-wsa**GrG/z$_2$**a-AEEA	820.37	1163.58, 1164.53, 1198.55
27-2	Ac-AEEA-wsa**GlG/z$_2$**a-AEEA	777.35	1120.57, 1121.51, 1155.53
28-1	Ac-AEEA-wsa**GkG/z$_2$**a-AEEA	792.36	1135.58, 1136.52, 1170.55
28-2	Ac-AEEA-wsa**GyG/z$_2$**a-AEEA	827.33	1170.55, 1171.49, 1205.51
29-1	Ac-AEEA-wsa**lG/lG**a-AEEA	720.33	1105.56
29-2	Ac-AEEA-wsa**lGlG/**a-AEEA	890.44	1105.56
30-1	Ac-AEEA-wsa**yG/z$_1$G**a-AEEA	770.31	1198.55, 1157.48, 1155.53
30-2	Ac-AEEA-wsa**yGlG/**a-AEEA	940.42	1155.54
30-3	Ac-AEEA-wsa**yGrG/**a-AEEA	983.43	1198.56
30-4	Ac-AEEA-wsa**eGlG/**a-AEEA	906.40	1121.52
31-1	Ac-AEEA-wsa**lG/yG**a-AEEA	720.33	1155.54
31-2	Ac-AEEA-wsa**lGyG/**a-AEEA	940.41	1155.53
31-3	Ac-AEEA-wsa**rGyG/**a-AEEA	983.43	1198.55
31-4	Ac-AEEA-wsa**eGyG/**a-AEEA	942.35	1157.48
32-1	Ac-AEEA-wsa**yG/yG/**a-AEEA	770.31, 990.39	1205.52
32-2	Ac-AEEA-wsa**yG/kG/**a-AEEA	770.31, 955.42	1170.55
32-3	Ac-AEEA-wsa**kGyG/**a-AEEA	955.42	1170.55

Slash denotes the cleavage site by papain.

Supplementary Table S7. Sequences that were individually synthesized for papain validation.

	Sequence	37℃, 36 h
33	Ac-AEEA-wsa**k**a-AEEA	0%
34	Ac-AEEA-wsa**e**a-AEEA	0%
35	Ac-AEEA-wsa**y**a-AEEA	0%
36	Ac-AEEA-wsa**Gk**a-AEEA	0%
37	Ac-AEEA-wsa**Ge**a-AEEA	0%
38	Ac-AEEA-wsa**G/y**a-AEEA	2%
39	Ac-AEEA-wsa**kG**a-AEEA	0%
40	Ac-AEEA-wsa**eG**a-AEEA	0%
41	Ac-AEEA-wsa**yG/**a-AEEA	13%
42	Ac-AEEA-wsa**GkG**a-AEEA	0%
43	Ac-AEEA-wsa**GeG**a-AEEA	0%
44	Ac-AEEA-wsa**GyG/**a-AEEA	22%
45	Ac-AEEA-wsa**GGk**Ga-AEEA	0%
46	Ac-AEEA-wsa**GGe**Ga-AEEA	0%
47	Ac-AEEA-wsa**GGyG/**a-AEEA	24%
48	Ac-AEEA-wsa**yG/k**a-AEEA	5%
49	Ac-AEEA-wsa**yG/e**a-AEEA	3%
50	Ac-AEEA-wsa**yG/y**a-AEEA	40%
51	Ac-AEEA-wsa**kGyG/**a-AEEA	3%
52	Ac-AEEA-wsa**eGyG/**a-AEEA	20%
53	Ac-AEEA-wsa**yGyG/**a-AEEA	92%
54	Ac-AEEA-wsa**yGkG/**a-AEEA	6%
55	Ac-AEEA-wsa**yGeG/**a-AEEA	4%
56	Ac-AEEA-wsa**GkG/y**a-AEEA	5%
57	Ac-AEEA-wsa**GeGy**a-AEEA	0%
58	Ac-AEEA-wsa**GyG/y**a-AEEA	53%
59	Ac-AEEA-wsa**GyG/k**a-AEEA	11%
60	Ac-AEEA-wsa**GyG/e**a-AEEA	5%
61	Ac-AEEA-wsa**GlGk**a-AEEA	0%
62	Ac-AEEA-wsa**GlGe**a-AEEA	0%
63	Ac-AEEA-wsa**GlG/y**a-AEEA	28%

Slash denotes the cleavage site by papain. The highlighted sequences exhibited superior cleavage yields, and **peptide 53** was selected as a candidate for further optimization.

Supplementary Table S8. Positional scan with a single mutation at P2 based on peptide Ac-AEEA-wsayGyG/a-AEEA.

	P2 scan	37°C, 36 h	37°C, 6 h	37°C, 1 h
64	Ac-AEEA-wsa**yGrG**/a-AEEA	16%		
65	Ac-AEEA-wsa**yGhG**/a-AEEA	0%		
66	Ac-AEEA-wsa**yGkG**/a-AEEA	6%		
67	Ac-AEEA-wsa**yGeG**/a-AEEA	4%		
68	Ac-AEEA-wsa**yGsG**/a-AEEA	0%		
69	Ac-AEEA-wsa**yGqG**/a-AEEA	0%		
70	Ac-AEEA-wsa**yGpG**/a-AEEA	0%		
71	Ac-AEEA-wsa**yGaG**/a-AEEA	0%		
72	Ac-AEEA-wsa**yGvG**/a-AEEA	18%		
73	Ac-AEEA-wsa**yGlG**/a-AEEA	16%	3%	
74	Ac-AEEA-wsa**yGfG**/a-AEEA	98%	25%	17%
75	Ac-AEEA-wsa**yGwG**/a-AEEA	98%	95%	21%
76	Ac-AEEA-wsa**yGyG**/a-AEEA	92%	24%	7%

Slash denotes the cleavage site by papain.

Supplementary Table S9. Positional scan with a single mutation at P1' based on peptide Ac-AEEA-sayGwG/a-AEEA.

	P1' scan	37°C, 36 h	37°C, 6 h	37°C, 1 h
77	Ac-AEEA-sa**yGwG/h**-AEEA	82%		17%
78	Ac-AEEA-sa**yGwG/k**-AEEA	20%		
79	Ac-AEEA-sa**yGwG/e**-AEEA	26%		
80	Ac-AEEA-sa**yGwG/s**-AEEA	61%		
81	Ac-AEEA-sa**yGwG/q**-AEEA	47%		
82	Ac-AEEA-sa**yGwG/p**-AEEA	0%		
83	Ac-AEEA-sa**yGwG/a**-AEEA	84%	54%	18%
84	Ac-AEEA-sa**yGwG/v**-AEEA	29%		
85	Ac-AEEA-sa**yGwG/l**-AEEA	98%	75%	20%
86	Ac-AEEA-sa**yGwG/f**-AEEA	98%	69%	19%
87	Ac-AEEA-sa**yGwG/w**-AEEA	98%	71%	21%
88	Ac-AEEA-sa**yGwG/y**-AEEA	98%	86%	23%

Slash denotes the cleavage site by papain.

Supplementary Table S10. Positional scan with a single mutation at P1 based on peptide Ac-AEEA-sayGwG/a-AEEA.

	P1 scan	37°C, 36 h
89	Ac-AEEA-sa**y**G**w**h/a-AEEA	0%
90	Ac-AEEA-sa**y**G**w**k/a-AEEA	0%
91	Ac-AEEA-sa**y**G**w**e/a-AEEA	0%
92	Ac-AEEA-sa**y**G**w**s/a-AEEA	0%
93	Ac-AEEA-sa**y**G**w**q/a-AEEA	0%
94	Ac-AEEA-sa**y**G**w**p/a-AEEA	0%
95	Ac-AEEA-sa**y**G**w**a/a-AEEA	0%
96	Ac-AEEA-sa**y**G**w**v/a-AEEA	0%
97	Ac-AEEA-sa**y**G**w**l/a-AEEA	0%
98	Ac-AEEA-sa**y**G**w**f/a-AEEA	0%
99	Ac-AEEA-sa**y**G**w**w/a-AEEA	0%
100	Ac-AEEA-sa**y**G**w**y/a-AEEA	0%

Slash denotes the cleavage site by papain.

Supplementary Table S11. Positional scan with a single mutation at P3 based on peptide Ac-AEEA-sayGwG/a-AEEA.

	P3 scan	37°C, 36 h	37°C, 1 h
101	Ac-AEEA-sa**y**h**w**G/a-AEEA	0%	
102	Ac-AEEA-sa**y**k**w**G/a-AEEA	0%	
103	Ac-AEEA-sa**y**e**w**G/a-AEEA	0%	
104	Ac-AEEA-sa**y**s**w**G/a-AEEA	28%	2%
105	Ac-AEEA-sa**y**q**w**G/a-AEEA	0%	
106	Ac-AEEA-sa**y**p**w**G/a-AEEA	1%	
107	Ac-AEEA-sa**y**a**w**G/a-AEEA	42%	2%
108	Ac-AEEA-sa**y**v**w**G/a-AEEA	2%	
109	Ac-AEEA-sa**y**l**w**G/a-AEEA	<1%	
110	Ac-AEEA-sa**y**f**w**G/a-AEEA	<1%	
111	Ac-AEEA-sa**y**w**w**G/a-AEEA	<1%	
112	Ac-AEEA-sa**y**y**w**G/a-AEEA	<1%	

Slash denotes the cleavage site by papain.

Supplementary Table S12. Positional scan with a single mutation at P4 based on peptide Ac-AEEA-sayGwG/a-AEEA.

	P4 scan	37°C, 36 h	37°C, 6 h	37°C, 1 h
113	Ac-AEEA-saG**GwG/**a-AEEA	6%		
114	Ac-AEEA-sah**GwG/**a-AEEA	32%		
115	Ac-AEEA-sak**GwG/**a-AEEA	3%		
116	Ac-AEEA-sae**GwG/**a-AEEA	28%		
117	Ac-AEEA-sas**GwG/**a-AEEA	20%		
118	Ac-AEEA-saq**GwG/**a-AEEA	12%		
119	Ac-AEEA-sap**GwG/**a-AEEA	30%		
120	Ac-AEEA-saa**GwG/**a-AEEA	18%		
121	Ac-AEEA-sal**GwG/**a-AEEA	33%		
122	Ac-AEEA-sal**GwG/**a-AEEA	30%		
123	Ac-AEEA-saf**GwG/**a-AEEA	98%	77%	17%
124	Ac-AEEA-saw**GwG/**a-AEEA	96%	71%	10%

Slash denotes the cleavage site by papain.

Supplementary Table S13. Positional scan with a single mutation at P5 based on peptide Ac-AEEA-sayGwG/a-AEEA.

	P5 scan	37°C, 36 h	37°C, 6 h	37°C, 1 h
125	Ac-AEEA-sG**yGwG/**a-AEEA	84%		8%
126	Ac-AEEA-sh**yGwG/**a-AEEA	81%		8%
127	Ac-AEEA-sk**yGwG/**a-AEEA	76%		9%
128	Ac-AEEA-se**yGwG/**a-AEEA	96%	41%	10%
129	Ac-AEEA-ss**yGwG/**a-AEEA	69%		8%
130	Ac-AEEA-sq**yGwG/**a-AEEA	89%		11%
131	Ac-AEEA-sp**yGwG/**a-AEEA	99%	97%	41%
132	Ac-AEEA-sv**yGwG/**a-AEEA	99%	94%	37%
133	Ac-AEEA-sl**yGwG/**a-AEEA	98%	99%	48%
134	Ac-AEEA-sf**yGwG/**a-AEEA	98%	97%	27%
135	Ac-AEEA-sw**yGwG/**a-AEEA	98%	94%	25%
136	Ac-AEEA-sy**yGwG/**a-AEEA	98%	66%	13%

Slash denotes the cleavage site by papain.

Supplementary Table S14. Sequences included in the test.

	P1'-P5 combination	Sequence	37°C, 1 h
137	P5 = l, AEEA2	Ac-AEEA2-s**l**y**G**w**G**/**a**-AEEA	40%
138	P5 = l, P1' = y	Ac-AEEA2-s**l**y**G**w**G**/**y**-AEEA	53%
139	P5 = l, P1' = l	Ac-AEEA2-s**l**y**G**w**G**/**l**-AEEA	37%
140	P5 = l, P1 = a	Ac-AEEA2-s**l**y**G**w**a**/**a**-AEEA	0%
141	P5 = l, P3 = a	Ac-AEEA2-s**l**y**a**w**G**/**a**-AEEA	3%
142	P5 = l, P4 = f	Ac-AEEA2-s**l**f**G**w**G**/**a**-AEEA	18%
143	P5 = l, P4 = w	Ac-AEEA2-s**l**w**G**w**G**/**a**-AEEA	15%
144	P5 = l, P1' = y, P1 = a	Ac-AEEA2-s**l**y**G**w**a**/**y**-AEEA	0%
145	P5 = l, P1' = y, P3 = a	Ac-AEEA2-s**l**y**a**w**G**/**y**-AEEA	9%
146	P5 = l, P3 = a, P4 = f	Ac-AEEA2-s**l**f**a**w**G**/**a**-AEEA	2%
147	P5 = l, P1'=y, P3=a, P4=f	Ac-AEEA2-s**l**f**a**w**G**/**y**-AEEA	6%

Slash denotes the cleavage site by papain. AEEA2 (AEEA-AEEA) was used to facilitate HPLC analysis.

Supplementary Table S15. Positional scan with a single mutation at P6 based on peptide Ac-AEEA2-lyGwG/y-AEEA .

	P6 scan	37°C, 1 h
148	Ac-AEEA2-lyGwG/y-AEEA	41%
149	Ac-AEEA2-GlyGwG/y-AEEA	32%
150	Ac-AEEA2-rlyGwG/y-AEEA	23%
151	Ac-AEEA2-hlyGwG/y-AEEA	42%
152	Ac-AEEA2-klyGwG/y-AEEA	20%
153	Ac-AEEA2-elyGwG/y-AEEA	42%
154	Ac-AEEA2-slyGwG/y-AEEA	56%
155	Ac-AEEA2-qlyGwG/y-AEEA	48%
156	Ac-AEEA2-plyGwG/y-AEEA	31%
157	Ac-AEEA2-alyGwG/y-AEEA	31%
158	Ac-AEEA2-llyGwG/y-AEEA	33%
159	Ac-AEEA2-flyGwG/y-AEEA	44%
160	Ac-AEEA2-wlyGwG/y-AEEA	39%
161	Ac-AEEA2-ylyGwG/y-AEEA	40%

Slash denotes the cleavage site by papain.

Supplementary Table S16. Positional scan with a single mutation at P2' based on peptide Ac-AEEA2-lyGwG/y-AEEA .

	P2' scan	37°C, 1 h
162	Ac-AEEA2-lyGwG/yr-AEEA	35%
163	Ac-AEEA2-lyGwG/yh-AEEA	31%
164	Ac-AEEA2-lyGwG/yk-AEEA	24%
165	Ac-AEEA2-lyGwG/ye-AEEA	26%
166	Ac-AEEA2-lyGwG/ys-AEEA	40%
167	Ac-AEEA2-lyGwG/yq-AEEA	30%
168	Ac-AEEA2-lyGwG/yp-AEEA	24%
169	Ac-AEEA2-lyGwG/ya-AEEA	38%
170	Ac-AEEA2-lyGwG/yl-AEEA	61%
171	Ac-AEEA2-lyGwG/yf-AEEA	61%
172	Ac-AEEA2-lyGwG/yw-AEEA	73%
173	Ac-AEEA2-lyGwG/yy-AEEA	72%
	P6 and P2' combination	**37°C, 1 h**
174	Ac-AEEA2-slyGwG/yw-AEEA	75%
175	Ac-AEEA2-slyGwG/yy-AEEA	77%
176	Ac-AEEA2-qlyGwG/yw-AEEA	83%
177	Ac-AEEA2-qlyGwG/yy-AEEA	82%

Slash denotes the cleavage site by papain.

Supplementary Table S17. Sequences included in -wsaGGjGG- and -wsaGjjGG-.

	Sequence		Sequence
B1	Ac-AEEA-wsaG**Gj_1**GG-AEEA	**B7**	Ac-AEEA-wsa**Gj_2j_1**GG-AEEA
B2	Ac-AEEA-wsaG**Gj_2**GG-AEEA	**B8**	Ac-AEEA-wsa**Gj_2j_2**GG-AEEA
B3	Ac-AEEA-wsaG**Gj_3**GG-AEEA	**B9**	Ac-AEEA-wsa**Gj_2j_3**GG-AEEA
B4	Ac-AEEA-wsa**Gj_1j_1**GG-AEEA	**B10**	Ac-AEEA-wsa**Gj_3j_1**GG-AEEA
B5	Ac-AEEA-wsa**Gj_1j_2**GG-AEEA	**B11**	Ac-AEEA-wsa**Gj_3j_2**GG-AEEA
B6	Ac-AEEA-wsa**Gj_1j_3**GG-AEEA	**B12**	Ac-AEEA-wsa**Gj_3j_3**GG-AEEA

j_1 = h + a + p + y

j_2 = r + s + d + f

j_3 = k + e + v + w

j = j_1 + j_2 + j_3

-wsa- and -as- were appended to the N-terminus of the target screening sequences to ensure successful HPLC analysis of the peptide cleavage. These appended D-peptides also ensure that any observed proteolytic activity results from recognizing D-peptide substrates. This principle was applied to subsequent experiments. All samples were incubated at 37°C for 36 hours.

Supplementary Table S18. Observed sequences recognized by hCATB based on Library #3.

	Sequence	37℃, 36 h
B1-1	Ac-AEEA-wsaG**Gy**G/G-AEEA	10%
B1-2	Ac-AEEA-wsaG**Gh**G/G-AEEA	3%
B2-1	Ac-AEEA-wsaG**Gf**G/G-AEEA	10%
B2-2	Ac-AEEA-wsaG**Gr**G/G-AEEA	3%
B3-1	Ac-AEEA-wsaG**Gw**G/G-AEEA	11%
B4-1	Ac-AEEA-wsa**Gyy**G/G-AEEA	8%
B4-2	Ac-AEEA-wsa**Gpy**G/G-AEEA	5%
B5-1	Ac-AEEA-wsa**Gyf**G/G-AEEA	6%
B5-2	Ac-AEEA-wsa**Gaf**G/G-AEEA	4%
B5-3	Ac-AEEA-wsa**Gpf**G/G-AEEA	4%
B6-1	Ac-AEEA-wsa**Gyw**G/G-AEEA	6%
B6-2	Ac-AEEA-wsa**Gaw**G/G-AEEA	7%
B6-3	Ac-AEEA-wsa**Gpw**G/G-AEEA	5%
B6-4	Ac-AEEA-wsa**Gpv**G/G-AEEA	5%
B7-1	Ac-AEEA-wsa**Gfy**G/G-AEEA	12%
B7-2	Ac-AEEA-wsa**Gfp**G/G-AEEA	5%
B7-3	Ac-AEEA-wsa**Gry**G/G-AEEA	4%
B7-4	Ac-AEEA-wsa**Gsy**G/G-AEEA	4%
B7-5	Ac-AEEA-wsa**Gsh**G/G-AEEA	3%
B8-1	Ac-AEEA-wsa**Gff**G/G-AEEA	5%
B9-1	Ac-AEEA-wsa**Gfw**G/G-AEEA	7%
B9-2	Ac-AEEA-wsa**Gsw**G/G-AEEA	3%
B9-3	Ac-AEEA-wsa**Grw**G/G-AEEA	3%
B10-1	Ac-AEEA-wsa**Gwy**G/G-AEEA	6%
B10-2	Ac-AEEA-wsa**Gwp**G/G-AEEA	5%

Slash denotes the cleavage site by hCATB. The concentration of hCATB was 20 nM, and of substrates was 40 μM. Only sequences with more than 5% cleavage products of B1-B12 are presented. The D-amino acid, highlighted in red, was recognized as P2 or P3, while Gly was P1.

Supplementary Table S19. Sequences that were individually synthesized for validation.

	Sequence	37℃, 24 h
B1-1	Ac-AEEA-wsaG**GyG**/G-AEEA	40%
B2-1	Ac-AEEA-wsaG**GfG**/G-AEEA	31%
B3-1	Ac-AEEA-wsaG**GwG**/G-AEEA	35%
B4-1	Ac-AEEA-wsa**GyyG**/G-AEEA	55%
B5-1	Ac-AEEA-wsa**GyfG**/G-AEEA	44%
B6-1	Ac-AEEA-wsa**GywG**/G-AEEA	43%
B6-2	Ac-AEEA-wsa**GawG**/G-AEEA	32%
B6-3	Ac-AEEA-wsa**GpwG**/G-AEEA	6%
B6-4	Ac-AEEA-wsa**GpvG**/G-AEEA	10%
B7-1	Ac-AEEA-wsa**GfyG**/G-AEEA	34%
B7-2	Ac-AEEA-wsa**GfpG**/G-AEEA	21%
B8-1	Ac-AEEA-wsa**GffG**/G-AEEA	31%
B9-1	Ac-AEEA-wsa**GfwG**/G-AEEA	35%
B10-1	Ac-AEEA-wsa**GwyG**/G-AEEA	39%
B10-2	Ac-AEEA-wsa**GwpG**/G-AEEA	28%

Slash denotes the cleavage site by hCATB.

Supplementary Table S20. Positional scan with a single mutation at P2 based on peptide Ac-AEEA2-GGxG/G-AEEA.

	P2 scan	37℃, 12 h
B13	Ac-AEEA2-GG**rG**/G-AEEA	19%
B14	Ac-AEEA2-GG**hG**/G-AEEA	19%
B15	Ac-AEEA2-GG**kG**/G-AEEA	12%
B16	Ac-AEEA2-GG**eG**/G-AEEA	1%
B17	Ac-AEEA2-GG**sG**/G-AEEA	6%
B18	Ac-AEEA2-GG**qG**/G-AEEA	1%
B19	Ac-AEEA2-GG**pG**/G-AEEA	2%
B20	Ac-AEEA2-GG**aG**/G-AEEA	1%
B21	Ac-AEEA2-GG**lG**/G-AEEA	3%
B22	Ac-AEEA2-GG**fG**/G-AEEA	15%
B23	Ac-AEEA2-GG**wG**/G-AEEA	17%
B24	Ac-AEEA2-GG**yG**/G-AEEA	20%

Slash denotes the cleavage site by hCATB.

Supplementary Table S21. Positional scan with a single mutation at P3 based on peptide Ac-AEEA2-GGyG/G-AEEA.

	P3 scan	37°C, 12 h
B25	Ac-AEEA2-G**r**yG/G-AEEA	11%
B26	Ac-AEEA2-G**h**yG/G-AEEA	24%
B27	Ac-AEEA2-G**k**yG/G-AEEA	13%
B28	Ac-AEEA2-G**e**yG/G-AEEA	4%
B29	Ac-AEEA2-G**s**yG/G-AEEA	17%
B30	Ac-AEEA2-G**q**yG/G-AEEA	11%
B31	Ac-AEEA2-G**p**yG/G-AEEA	4%
B32	Ac-AEEA2-G**a**yG/G-AEEA	30%
B33	Ac-AEEA2-G**l**yG/G-AEEA	8%
B34	Ac-AEEA2-G**f**yG/G-AEEA	19%
B35	Ac-AEEA2-G**w**yG/G-AEEA	19%
B36	Ac-AEEA2-G**y**yG/G-AEEA	30%

Slash denotes the cleavage site by hCATB.

Supplementary Table S22. Positional scan with a single mutation at P4, P5 based on peptide Ac-AEEA2-GGyyG/G-AEEA.

	P4 scan	37°C, 12 h
B37	Ac-AEEA2-G**G**yyG/G-AEEA	29%
B38	Ac-AEEA2-G**r**yyG/G-AEEA	70%
B39	Ac-AEEA2-G**h**yyG/G-AEEA	76%
B40	Ac-AEEA2-G**k**yyG/G-AEEA	71%
B41	Ac-AEEA2-G**e**yyG/G-AEEA	20%
B42	Ac-AEEA2-G**s**yyG/G-AEEA	36%
B43	Ac-AEEA2-G**q**yyG/G-AEEA	39%
B44	Ac-AEEA2-G**p**yyG/G-AEEA	44%
B45	Ac-AEEA2-G**a**yyG/G-AEEA	40%
B46	Ac-AEEA2-G**l**yyG/G-AEEA	45%
B47	Ac-AEEA2-G**f**yyG/G-AEEA	69%
B48	Ac-AEEA2-G**w**yyG/G-AEEA	60%
B49	Ac-AEEA2-G**y**yyG/G-AEEA	53%
	P5 scan	**37°C, 12 h**
B50	Ac-AEEA2-**r**GyyG/G-AEEA	94%
B51	Ac-AEEA2-**h**GyyG/G-AEEA	70%
B52	Ac-AEEA2-**k**GyyG/G-AEEA	71%
B53	Ac-AEEA2-**e**GyyG/G-AEEA	15%
B54	Ac-AEEA2-**s**GyyG/G-AEEA	33%
B55	Ac-AEEA2-**q**GyyG/G-AEEA	34%
B56	Ac-AEEA2-**p**GyyG/G-AEEA	41%
B57	Ac-AEEA2-**a**GyyG/G-AEEA	31%
B58	Ac-AEEA2-**l**GyyG/G-AEEA	67%
B59	Ac-AEEA2-**f**GyyG/G-AEEA	68%
B60	Ac-AEEA2-**w**GyyG/G-AEEA	68%
B61	Ac-AEEA2-**y**GyyG/G-AEEA	55%

Slash denotes the cleavage site by hCATB.

Supplementary Table S23. Positional scan with a single mutation at P1' based on peptide Ac-AEEA-rkyyG/-AEEA.

	P1' scan	37°C, 1 h
B62	Ac-AEEA2-**rkyyG/r**-AEEA	0%
B63	Ac-AEEA2-**rkyyG/h**-AEEA	6%
B64	Ac-AEEA2-**rkyyG/k**-AEEA	0%
B65	Ac-AEEA2-**rkyyG/e**-AEEA	19%
B66	Ac-AEEA2-**rkyyG/s**-AEEA	3%
B67	Ac-AEEA2-**rkyyG/q**-AEEA	8%
B68	Ac-AEEA2-**rkyyG/p**-AEEA	0%
B69	Ac-AEEA2-**rkyyG/a**-AEEA	3%
B70	Ac-AEEA2-**rkyyG/l**-AEEA	48%
B71	Ac-AEEA2-**rkyyG/f**-AEEA	71%
B72	Ac-AEEA2-**rkyyG/w**-AEEA	80%
B73	Ac-AEEA2-**rkyyG/y**-AEEA	71%

Slash denotes the cleavage site by hCATB.

Supplementary Table S24. Combination of preferred amino acids at P3 and P4.

	P3-P4 combination	37°C, 6 h	37°C, 1 h
B74	Ac-AEEA2-**rrhyG/**G-AEEA	87%	17%
B75	Ac-AEEA2-**rryyG/**G-AEEA	94%	26%
B76	Ac-AEEA2-**rhhyG/**G-AEEA	59%	7%
B77	Ac-AEEA2-**rhyyG/**G-AEEA	81%	13%
B78	Ac-AEEA2-**rkhyG/**G-AEEA	56%	7%
B79	Ac-AEEA2-**rkyyG/**G-AEEA	90%	24%
B80	Ac-AEEA2-**rfhyG/**G-AEEA	38%	4%
B81	Ac-AEEA2-**rfyyG/**G-AEEA	62%	9%

Slash denotes the cleavage site by hCATB.

Supplementary Table S25. X-ray structure statistics of Ac-rkyyG-hCATB complex.

Ac-rkyyG-hCATB (9XHR)	
Wavelength	1.5418
Resolution range	30.78 - 1.5 (1.554 - 1.5)
Space group	P 21 21 21
Unit cell	31.0984 81.3307 94.2191 90 90 90
Total reflections	223427 (14062)
Unique reflections	39111 (3823)
Multiplicity	5.7 (3.7)
Completeness (%)	99.70 (98.79)
Mean I/sigma (I)	18.48 (4.09)
Wilson B-factor	9.89
R-merge	0.05729 (0.2362)
R-meas	0.06207 (0.275)
R-pim	0.02309 (0.1371)
CC1/2	0.999 (0.935)
CC*	1 (0.983)
Reflections used in refinement	39106 (3823)
Reflections used for R-free	2003 (195)
R-work	0.1708 (0.1895)
R-free	0.1944 (0.2048)
CC (work)	0.956 (0.851)
CC (free)	0.950 (0.872)
Number of non-hydrogen atoms	2407
macromolecules	2001
ligands	52
solvent	354
Protein residues	255
RMS (bonds)	0.398
RMS (angles)	5.68
Ramachandran favored (%)	96.84
Ramachandran allowed (%)	3.16
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	3.72
Clashscore	20.89
Average B-factor	14.98
macromolecules	11.59
ligands	40.34
solvent	30.39

Supplementary Table S26. X-ray structure statistics of Ac-YYG-hCATB complex.

Ac-YYG-hCATB (9VA8)	
Wavelength	1.5418
Resolution range	28.35 - 1.5 (1.554 - 1.5)
Space group	P 21 21 21
Unit cell	30.6169 75.096 97.6422 90 90 90
Total reflections	169204 (10587)
Unique reflections	36878 (3545)
Multiplicity	4.6 (3.0)
Completeness (%)	99.64 (97.68)
Mean I/sigma (I)	23.98 (2.40)
Wilson B-factor	8.82
R-merge	0.0836 (0.3868)
R-meas	0.09278 (0.4687)
R-pim	0.03863 (0.2588)
CC1/2	0.992 (0.788)
CC*	0.998 (0.939)
Reflections used in refinement	36874 (3543)
Reflections used for R-free	1784 (180)
R-work	0.1892 (0.2571)
R-free	0.2200 (0.2878)
CC (work)	0.956 (0.676)
CC (free)	0.947 (0.685)
Number of non-hydrogen atoms	2189
macromolecules	1949
ligands	32
solvent	208
Protein residues	254
RMS (bonds)	0.302
RMS (angles)	6.77
Ramachandran favored (%)	96.43
Ramachandran allowed (%)	3.57
Ramachandran outliers (%)	0.00
Rotamer outliers (%)	1.45
Clashscore	1.32
Average B-factor	12.00
macromolecules	10.94
ligands	12.33
solvent	21.90

Supplementary Table S27. hCATB cleavage of D-peptide sequences with a "spacer" at the C-terminus.

	Sequence	37℃, 0.5 h	37℃, 10 min
B82	Ac-AEEA2-**rrhyG/**-spacer-AEEA	49%	
B83	Ac-AEEA2-**rryyG/**-spacer-AEEA	94%	57%
B84	Ac-AEEA2-**rhhyG/**-spacer-AEEA	34%	
B85	Ac-AEEA2-**rhyyG/**-spacer-AEEA	94%	42%
B86	Ac-AEEA2-**rkhyG/**-spacer-AEEA	48%	
B87	Ac-AEEA2-**rkyyG/**-spacer-AEEA	100%	65%
B88	Ac-AEEA2-**rfhyG/**-spacer-AEEA	28%	
B89	Ac-AEEA2-**rfyyG/**-spacer-AEEA	92%	

Slash denotes the cleavage site by hCATB. For the application of antibody-drug conjugates (ADCs), the C-terminus of D-peptides was appended with 3-(4-Aminophenyl) propionic acid, a substitute for the self-immolative ρ-aminobenzyl carbamate (PAB) spacer. The "spacer" refers to 3-(4-Aminophenyl) propionic acid. Sequences were synthesized and compared based on **Supplementary Table S24**.

Supplementary Table S28. In vitro stability of anti-HER2 ADCs in plasma from different species.

Human Plasma (n = 3)					
	Time (d)	ADC (μg/mL)	mAb (μg/mL)	ADC/mAb (%)	relative residual ADC (%)
ADC-rkyyG-MMAE	0.00	0.269	0.953	28.2	100.0
	0.17	0.266	1.000	26.6	94.3
	1.00	0.246	1.073	22.9	81.2
	3.00	0.262	1.093	23.9	84.9
	8.00	0.176	0.791	22.3	79.0
	14.00	0.160	0.782	20.5	72.7
	28.00	0.171	0.851	20.1	71.3
ADC-VC-MMAE	0.00	0.280	0.731	38.4	100.0
	0.17	0.255	0.732	34.9	91.0
	1.00	0.226	0.741	30.6	79.7
	3.00	0.196	0.686	28.5	74.4
	8.00	0.151	0.723	20.8	54.3
	14.00	0.138	0.651	21.2	55.2
	28.00	0.122	0.767	15.9	41.3
Mouse Plasma (n = 3)					
	Time (d)	ADC (μg/mL)	mAb (μg/mL)	ADC/mAb (%)	relative residual ADC (%)
ADC-rkyyG-MMAE	0.00	0.447	0.349	128.0	100.0
	0.17	0.427	0.375	113.9	89.0
	1.00	0.350	0.365	95.9	74.9
	3.00	0.294	0.349	84.2	65.8
	8.00	0.289	0.331	87.4	68.3
	14.00	0.248	0.288	86.2	67.4
ADC-VC-MMAE	0.00	0.280	0.289	96.7	100.0
	0.17	0.050	0.306	16.5	17.0
	1.00	0.035	0.314	11.1	11.5
	3.00	0.031	0.310	10.1	10.5
	8.00	0.034	0.298	11.6	12.0
	14.00	0.032	0.229	14.0	14.4
Rat Plasma (n = 3)					
	Time (d)	ADC (μg/mL)	mAb (μg/mL)	ADC/mAb (%)	relative residual ADC (%)
ADC-rkyyG-MMAE	0.00	0.319	0.352	90.8	100.0
	0.17	0.288	0.341	84.6	93.2
	1.00	0.266	0.327	81.3	89.6
	3.00	0.231	0.303	76.3	84.1
	8.00	0.188	0.261	71.9	79.3
	14.00	0.163	0.211	77.0	84.9
ADC-VC-MMAE	0.00	0.279	0.369	75.7	100.0
	0.17	0.282	0.340	82.9	109.4
	1.00	0.238	0.326	73.0	96.4
	3.00	0.180	0.271	66.4	87.7
	8.00	0.111	0.200	55.6	73.3
	14.00	0.083	0.161	51.6	68.2

Supplementary Table S29. In vivo PK parameters for anti-HER2 ADCs in mice.

	$t_{1/2}$ total mAb (day)	$t_{1/2}$ ADC (day)	$AUC_{total\ mAb}$ (h*μg/mL)	AUC_{ADC} (h*μg/mL)
ADC-rkyyG-MMAE	13.46	7.05	173.81	89.92
ADC-VC-MMAE	28.40	0.31	188.54	5.78

Supplementary Table S30: Cleavage yield of substrates by cathepsin proteases.

Cathepsin	-rkyyG-	-Val-Cit-	-GGFG-
B	100%	100%	50%
A	0	100%	95%
C	0	100%	65%
L	0	100%	98%
S	0	60%	20%
V	0	100%	100%

All reactions were processed at 37°C for 12 hours. Details can be found in the **Supplementary methods**.

Supplementary Table S31. Sequences included in Library #3.

	Sequence
C1	Ac-AEEA-**rkyy**v_1-spacer-AEEA
C2	Ac-AEEA-**rkyy**v_2-spacer-AEEA
C3	Ac-AEEA-**rkyy**v_3-spacer-AEEA

v_1 = k + s + d + G + m + l + w

v_2 = n + h + e + a + t + y

v_3 = q + r + v + p + i + f

v = v_1 + v_2 + v_3

Supplementary Table S32. Sequences recognized by mCES1c based on Library #3.

	Sequence	37°C, 24 h
C1-1	Ac-AEEA-**rkyyG/**-spacer-AEEA	23%
C1-2	Ac-AEEA-**rkyyw/**-spacer-AEEA	77%
C2-1	Ac-AEEA-**rkyyy/**-spacer-AEEA	9%
C3-1	Ac-AEEA-**rkyyf/**-spacer-AEEA	41%

Slash denotes the cleavage site by mCES1c. The concentration of mCES1c was 33.7 nM, and of substrates was 40 μM. Only Gly, D-Trp, D-Tyr, and D-Phe could be recognized at P1 by mCES1c, with D-Trp being the most effective. This confirms that P1 is compatible with D-amino acids.

Supplementary Table S33. Positional scan with a single mutation at P2, P3 based on peptide Ac-AEEA-rkyyw-spacer-AEEA for mCES1c.

	P2 scan	37℃, 12 h
C4	Ac-AEEA-**rkyGw/**-spacer-AEEA	2%
C5	Ac-AEEA-**rkyrw/**-spacer-AEEA	8%
C6	Ac-AEEA-**rkyhw/**-spacer-AEEA	9%
C7	Ac-AEEA-**rkykw/**-spacer-AEEA	11%
C8	Ac-AEEA-**rkyew/**-spacer-AEEA	5%
C9	Ac-AEEA-**rkysw/**-spacer-AEEA	10%
C10	Ac-AEEA-**rkyqw/**-spacer-AEEA	6%
C11	Ac-AEEA-**rkypw/**-spacer-AEEA	4%
C12	Ac-AEEA-**rkyaw/**-spacer-AEEA	16%
C13	Ac-AEEA-**rkylw/**-spacer-AEEA	31%
C14	Ac-AEEA-**rkyfw/**-spacer-AEEA	14%
C15	Ac-AEEA-**rkyww/**-spacer-AEEA	5%
C16	Ac-AEEA-**rkyyw/**-spacer-AEEA	14%
	P3 scan	**37℃, 12 h**
C17	Ac-AEEA-**rkGyw/**-spacer-AEEA	0%
C18	Ac-AEEA-**rkryw/**-spacer-AEEA	0%
C19	Ac-AEEA-**rkhyw/**-spacer-AEEA	0%
C20	Ac-AEEA-**rkkyw/**-spacer-AEEA	0%
C21	Ac-AEEA-**rkeyw/**-spacer-AEEA	0%
C22	Ac-AEEA-**rksyw/**-spacer-AEEA	0%
C23	Ac-AEEA-**rkqyw/**-spacer-AEEA	0%
C24	Ac-AEEA-**rkpyw/**-spacer-AEEA	5%
C25	Ac-AEEA-**rkayw/**-spacer-AEEA	21%
C26	Ac-AEEA-**rklyw/**-spacer-AEEA	26%
C27	Ac-AEEA-**rkfyw/**-spacer-AEEA	7%
C28	Ac-AEEA-**rkwyw/**-spacer-AEEA	36%

Slash denotes the cleavage site by mCES1c.

Supplementary Table S34. Positional scan with a single mutation at P4, P5 based on peptide Ac-AEEA-rkwlw-spacer-AEEA for mCES1c .

	P4 scan	37°C, 1 h
C29	Ac-AEEA2-**rGwlw**-spacer-AEEA	5%
C30	Ac-AEEA2-**rrwlw**-spacer-AEEA	39%
C31	Ac-AEEA2-**rhwlw**-spacer-AEEA	15%
C32	Ac-AEEA2-**rkwlw**-spacer-AEEA	13%
C33	Ac-AEEA2-**rewlw**-spacer-AEEA	23%
C34	Ac-AEEA2-**rswlw**-spacer-AEEA	23%
C35	Ac-AEEA2-**rqwlw**-spacer-AEEA	51%
C36	Ac-AEEA2-**rpwlw**-spacer-AEEA	6%
C37	Ac-AEEA2-**rawlw**-spacer-AEEA	21%
C38	Ac-AEEA2-**rlwlw**-spacer-AEEA	94%
C39	Ac-AEEA2-**rfwlw**-spacer-AEEA	32%
C40	Ac-AEEA2-**rwwlw**-spacer-AEEA	19%
C41	Ac-AEEA2-**rywlw**-spacer-AEEA	15%
	P5 scan	**37°C, 12 h**
C42	Ac-AEEA2-**Gkwlw**-spacer-AEEA	53%
C43	Ac-AEEA2-**rkwlw**-spacer-AEEA	81%
C44	Ac-AEEA2-**hkwlw**-spacer-AEEA	95%
C45	Ac-AEEA2-**kkwlw**-spacer-AEEA	72%
C46	Ac-AEEA2-**ekwlw**-spacer-AEEA	40%
C47	Ac-AEEA2-**skwlw**-spacer-AEEA	59%
C48	Ac-AEEA2-**qkwlw**-spacer-AEEA	79%
C49	Ac-AEEA2-**pkwlw**-spacer-AEEA	76%
C50	Ac-AEEA2-**akwlw**-spacer-AEEA	67%
C51	Ac-AEEA2-**lkwlw**-spacer-AEEA	79%
C52	Ac-AEEA2-**fkwlw**-spacer-AEEA	83%
C53	Ac-AEEA2-**wkwlw**-spacer-AEEA	69%
C54	Ac-AEEA2-**ykwlw**-spacer-AEEA	97%
	P4-P5 combination	**37°C, 1 h**
C55	Ac-AEEA2-**rlwlw**-spacer-AEEA	94%
C56	Ac-AEEA2-**hlwlw**-spacer-AEEA	57%
C57	Ac-AEEA2-**flwlw**-spacer-AEEA	43%
C58	Ac-AEEA2-**ylwlw**-spacer-AEEA	65%

Slash denotes the cleavage site by mCES1c. P4 plays a crucial role in enhancing cleavage efficiency, with the reaction time set at 1 hour. The top reaction D-amino acids at P4 and P5 were combined, and −rlwlw− was identified as the best.

Supplementary Table S35. Single-point mutation scanning of the P2, P1, and P1' positions in the D-peptide substrate of hCATL.

	P1' scan	37°C, 24 h
L1	Ac-AEEA2-**rkyyG/r**-spacer-AEEA	5%
L2	Ac-AEEA2-**rkyyG/h**-spacer-AEEA	<1%
L3	Ac-AEEA2-**rkyyG/k**-spacer-AEEA	2%
L4	Ac-AEEA2-**rkyyG/e**-spacer-AEEA	4%
L5	Ac-AEEA2-**rkyyG/s**-spacer-AEEA	3%
L6	Ac-AEEA2-**rkyyG/q**-spacer-AEEA	2%
L7	Ac-AEEA2-**rkyyG/p**-spacer-AEEA	<1%
L8	Ac-AEEA2-**rkyyG/a**-spacer-AEEA	4%
L9	Ac-AEEA2-**rkyyG/l**-spacer-AEEA	10%
L10	Ac-AEEA2-**rkyyG/f**-spacer-AEEA	5%
L11	Ac-AEEA2-**rkyyG/w**-spacer-AEEA	9%
L12	Ac-AEEA2-**rkyyG/y**-spacer-AEEA	7%
	P1 scan	**37°C, 24 h**
L13	Ac-AEEA-**rkyyG/**-spacer-AEEA	49%
L14	Ac-AEEA-**rkyyh/**-spacer-AEEA	1%
L15	Ac-AEEA-**rkyys/**-spacer-AEEA	<1%
L16	Ac-AEEA-**rkyyp/**-spacer-AEEA	0%
L17	Ac-AEEA-**rkyya/**-spacer-AEEA	0%
L18	Ac-AEEA-**rkyyl/**-spacer-AEEA	<1%
L19	Ac-AEEA-**rkyyw/**-spacer-AEEA	0%
L20	Ac-AEEA-**rkyyy/**-spacer-AEEA	0%
	P2 scan	**37°C, 24 h**
L21	Ac-AEEA2-**GGrG/**-G-AEEA	3%
L22	Ac-AEEA2-**GGhG/**-G-AEEA	4%
L23	Ac-AEEA2-**GGkG/**-G-AEEA	1%
L24	Ac-AEEA2-**GGeG/**-G-AEEA	2%
L25	Ac-AEEA2-**GGsG/**-G-AEEA	<1%
L26	Ac-AEEA2-**GGqG/**-G-AEEA	<1%
L27	Ac-AEEA2-**GGpG/**-G-AEEA	<1%
L28	Ac-AEEA2-**GGaG/**-G-AEEA	<1%
L29	Ac-AEEA2-**GGlG/**-G-AEEA	3%
L30	Ac-AEEA2-**GGfG/**-G-AEEA	49%
L31	Ac-AEEA2-**GGwG/**-G-AEEA	50%
L32	Ac-AEEA2-**GGyG/**-G-AEEA	50%

Slash denotes the cleavage site by hCATL. The threshold for cleavage yield is set at 1%.

Supplementary Table S36. Single-point mutation scanning of the P5, P4, and P3 positions in the D-peptide substrate of hCATL.

	P3 scan	37°C, 24 h
L33	Ac-AEEA2-**GGyG/**-G-AEEA	20%
L34	Ac-AEEA2-**GryG/**-G-AEEA	6%
L35	Ac-AEEA2-**GhyG/**-G-AEEA	8%
L36	Ac-AEEA2-**GkyG/**-G-AEEA	9%
L37	Ac-AEEA2-**GeyG/**-G-AEEA	6%
L38	Ac-AEEA2-**GsyG/**-G-AEEA	7%
L39	Ac-AEEA2-**GqyG/**-G-AEEA	5%
L40	Ac-AEEA2-**GpyG/**-G-AEEA	11%
L41	Ac-AEEA2-**GayG/**-G-AEEA	26%
L42	Ac-AEEA2-**GlyG/**-G-AEEA	12%
L43	Ac-AEEA2-**GfyG/**-G-AEEA	11%
L44	Ac-AEEA2-**GwyG/**-G-AEEA	18%
L45	Ac-AEEA2-**GyyG/**-G-AEEA	9%
	P4 scan	**37°C, 24 h**
L46	Ac-AEEA2-**GGyyG/**-G-AEEA	12%
L47	Ac-AEEA2-**GryyG/**-G-AEEA	20%
L48	Ac-AEEA2-**GhyyG/**-G-AEEA	37%
L49	Ac-AEEA2-**GkyyG/**-G-AEEA	7%
L50	Ac-AEEA2-**GeyyG/**-G-AEEA	13%
L51	Ac-AEEA2-**GsyyG/**-G-AEEA	11%
L52	Ac-AEEA2-**GqyyG/**-G-AEEA	11%
L53	Ac-AEEA2-**GpyyG/**-G-AEEA	17%
L54	Ac-AEEA2-**GayyG/**-G-AEEA	13%
L55	Ac-AEEA2-**GlyyG/**-G-AEEA	22%
L56	Ac-AEEA2-**GfyyG/**-G-AEEA	34%
L57	Ac-AEEA2-**GwyyG/**-G-AEEA	55%
L58	Ac-AEEA2-**GyyyG/**-G-AEEA	29%
	P5 scan	**37°C, 24 h**
L59	Ac-AEEA2-**rGyyG**-G-AEEA	14%
L60	Ac-AEEA2-**hGyyG**-G-AEEA	17%
L61	Ac-AEEA2-**kGyyG**-G-AEEA	10%
L62	Ac-AEEA2-**eGyyG**-G-AEEA	11%
L63	Ac-AEEA2-**sGyyG**-G-AEEA	14%
L64	Ac-AEEA2-**pGyyG**-G-AEEA	15%
L65	Ac-AEEA2-**aGyyG**-G-AEEA	14%
L66	Ac-AEEA2-**lGyyG**-G-AEEA	17%
L67	Ac-AEEA2-**fGyyG**-G-AEEA	24%
L68	Ac-AEEA2-**wGyyG**-G-AEEA	25%
L69	Ac-AEEA2-**yGyyG**-G-AEEA	23%

Supplementary Videos

Supplementary Video S1

200 ns molecular dynamics simulation of hCATB in complex with the D-pentapeptide (–rkyyG–). The protease is shown in cartoon representation (gray), and the substrate is depicted as sticks (cyan). Water molecules and ions are omitted for clarity.

Supplementary Video S2

200 ns molecular dynamics simulation of hCATB in complex with the L-tripeptide (–YYG–). The protease is shown in cartoon representation (gray), and the substrate is depicted as sticks (cyan). Water molecules and ions are omitted for clarity.

Supplementary Video S3

200 ns molecular dynamics simulation of hCATB in complex with the D-tripeptide (–rkyyG–). The protease is shown in cartoon representation (gray), and the substrate is depicted as sticks (cyan). Water molecules and ions are omitted for clarity.

Supplementary Video S4

200 ns molecular dynamics simulation of papain in complex with the L-peptide (–LYGWGYW–). The protease is shown in cartoon representation (gray), and the substrate is depicted as sticks (cyan). Water molecules and ions are omitted for clarity.

Supplementary Video S5

200 ns molecular dynamics simulation of papain in complex with the D-peptide (–lyGwGyw–). The protease is shown in cartoon representation (gray), and the substrate is depicted as sticks (cyan). Water molecules and ions are omitted for clarity.

Supplementary Video S6

200 ns molecular dynamics simulation of mCES1c in complex with the L-peptide (–RLWLW–). The protease is shown in cartoon representation (gray), and the substrate is depicted as sticks (cyan). Water molecules and ions are omitted for clarity.

Supplementary Video S7

200 ns molecular dynamics simulation of mCES1c in complex with the D-peptide (–rlwlw–). The protease is shown in cartoon representation (gray), and the substrate is depicted as sticks (cyan). Water molecules and ions are omitted for clarity.

Supplementary Video S8

200 ns molecular dynamics simulation of mCES1c in complex with the D-peptide (–rlwlw–). At 40 ns into the simulation, the protease undergoes a pronounced conformational change, allowing the substrate to enter the active pocket. The protease is shown in cartoon representation (gray), and the substrate is depicted as sticks (cyan). Water molecules and ions are omitted for clarity.

References

51 Yoon, M. C., Hook, V., & O'Donoghue, A. J. Cathepsin B Dipeptidyl Carboxypeptidase and Endopeptidase Activities Demonstrated across a Broad pH Range. *Biochemistry*, **61**, 1904–1914 (2022).

52 Rawlings, N. D., Barrett, A. J., Thomas, P. D., Huang, X., Bateman, A., & Finn, R. D.. The MEROPS database of proteolytic enzymes, their substrates and inhibitors in 2017 and a comparison with peptidases in the PANTHER database. *Nucleic Acids Res.*,**46**, D624–D632 (2018).