Supporting Information

Strategic Alkene Incorporation into Peptide Backbones to Prevent Enzymatic Hydrolysis by Evading Molecular Recognition

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1. General Information

HPLC condition.
For HPLC separations, a Cosmosil 5C_{18}-AR-II analytical column (Nacalai Tesque, 4.6 × 250 mm, flow rate 1.0 mL min^{-1}), Cosmosil 5C_{18}-AR-II preparative column (Nacalai Tesque, 20 × 250 mm, flow rate 10 mL min^{-1}) was employed, and eluting products were detected by UV at 220 nm. A solvent system consisting of 0.1% TFA aqueous solution (v/v, solvent A) and 0.1% TFA in MeCN (v/v, solvent B) was used for HPLC elution.

Characterization data.
High-resolution mass spectra were recorded on Bruker Daltonics compact (ESI-MS) spectrometers in the positive and negative detection mode.

2. Experimental procedures of protein

(*E*)-Methylalkene-type Ub mimic (4):

On the Rink Amide ChemMatrix® resin (0.42 mmol/g loading), peptide was synthesized using standard Fmoc-based protocols (3.0 eq. each of amino acid containing Fmoc-Lys(Alloc)-OH using HATU (2.9 eq.) and DIPEA (3.0 eq.) in DMF (5 min) and Fmoc removal with 20% piperidine in DMF (5 min)). FITC was introduced at the *N*-terminus. Then, $Pd(PPh_3)_4$ (0.25 eq.) and $PhSiH_3$ (20.0 eq.) was applied to remove the Alloc group and release the ε-amino group of Lys. The resulting resin was washed three times each with the following solvents: 0.5% (v/v) DIPEA in DCM, 0.02 M sodium diethyldithiocarbamate in DMF and DMF. Then, Ns-Gly-ψ[(*E*)-CMe=CH]-Gly-OH (**2**) was introduced under HATU/DIPEA system. The Ns group was removed by the thiolate generated from K_2CO_3 (0.2 equiv.) in 5% (v/v) thiophenol/DMF for elongation. Note: To suppress the formation of aspartimide byproducts during solid-phase peptide synthesis, Fmoc-Gly(DMB)-OH was installed at the Gly53 residue. Then, the resin was washed with DMF×3, DCM×3, MeOH×3, Et_2O×3, and dried under reduced pressure. Dried resin was cleaved using a TFA cocktail (TFA/*m*-cresol/thioanisole/3,6-dioxa-1,8-octanedithiol/H_2O (85/5/5/2.5/2.5, (v/v))) at room temperature. After 2 h, cold Et_2O was added to the reaction mixture to give a precipitate. The resulting precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was purified by reversed-phase HPLC (25-45% solvent B over 90 min, 0.1% TFA, cosmosil 5C_{18}-AR-II 20×250 mm column) followed by lyophilization to yield (*E*)-methylalkene-type Ub mimic (**4**) (1.05 μmol, 2% yield). Analytical HPLC, t_R = 14.4 min (25-35% solvent B over 20 min); HRMS (ESI), Calcd for $C_{410}H_{660}N_{109}O_{123}S_2$ $[M+H]^+$ 9150, found 9150.

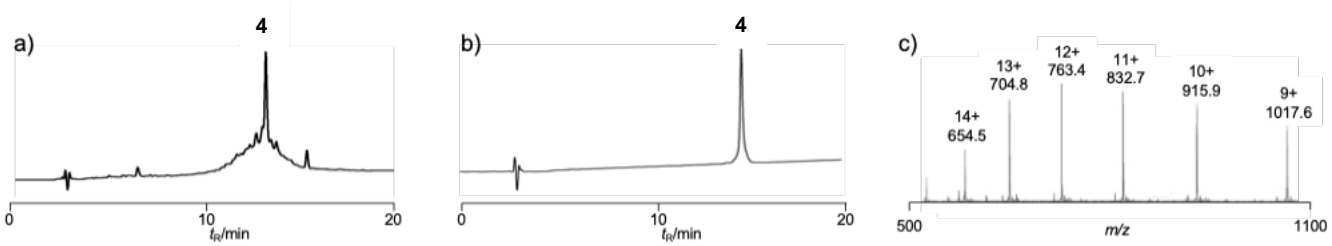

Figure S1: a) HPLC trace of crude material of **4**. b) HPLC trace of purified **4**. c) ESI-MS spectrum of purified **4**.

(*Z*)-Chloroalkene-type Ub mimic (7):

On the Rink Amide ChemMatrix® resin (0.42 mmol/g loading), peptide was synthesized using standard Fmoc-based protocols (3.0 eq. each of amino acid containing Fmoc-Lys(Alloc)-OH using HATU (2.9 eq.) and DIPEA (3.0 eq.) in DMF (5 min) and Fmoc removal with 20% piperidine in DMF (5 min)). FITC was introduced at the *N*-terminus. Then, $Pd(PPh_3)_4$ (0.25 eq.) and $PhSiH_3$ (20.0 eq.) was applied to remove the Alloc group and release the ε-NH_2 from Lys. The resulting resin was washed three times each with the following solvents: 0.5% (v/v) DIPEA in DCM, 0.02 M sodium diethyldithiocarbamate in DMF and DMF. After washing the resin, Fmoc-Gly-ψ[(*E*)-CCl=CH]-Gly-OH was conjugated by Fmoc-based protocols. Then, Fmoc removal with 20% piperidine in DMF (5 min)) for elongation of Fmoc-Amino acids of ubiquitin sequence. Note: To suppress the formation of aspartimide byproducts during solid-phase peptide synthesis, Fmoc-Gly(DMB)-OH was installed at the Gly53 residue. Then, the resin was washed with DMF×3, DCM×3, MeOH×3, Et_2O×3, and dried under reduced pressure. Dried resin was cleaved using a TFA cocktail (TFA/*m*-cresol/Thioanisole/3,6-Dioxa-1,8-Octanedithiol/H_2O (85/5/5/2.5/2.5, (v/v))) at room temperature. After 2 h, cold Et_2O was added to the reaction mixture to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was purified by reversed-phase HPLC (25-45% solvent B over 90 min, 0.1% TFA, cosmosil 5C_{18}-AR-II 20×250 mm column) followed by lyophilization to yield peptide **7** (1.20 μmol, 2% yield). Analytical HPLC, t_R = 15.8 min (25-35% solvent B over 20 min); HRMS (ESI), Calcd for $C_{409}H_{657}ClN_{109}O_{123}S_2$ $[M+H]^+$ 9170, found 9170.

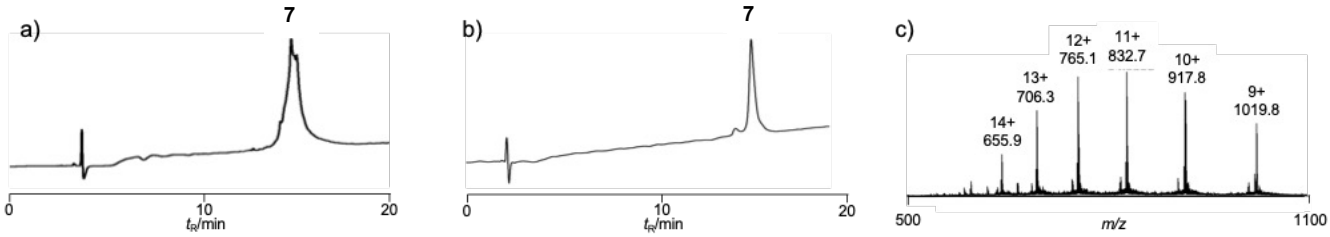

Figure S2: a) HPLC trace of crude material of **7**. b) HPLC trace of purified **7**. c) ESI-MS spectrum of purified **7**.

Native-type ubiquitin (8):

On the Rink Amide ChemMatrix® resin (0.42 mmol/g loading), peptide was synthesized using standard Fmoc-based protocols (3.0 eq. each of amino acid containing Fmoc-Lys(Alloc)-OH using HATU (2.9 eq.) and DIPEA (3.0 eq.) in DMF (5 min) and Fmoc removal with 20% piperidine in DMF (5 min)). FITC was introduced at the *N*-terminus. Then, $Pd(PPh_3)_4$ (0.25 eq.) and $PhSiH_3$ (20.0 eq.) was applied to remove the Alloc group and release the ε-amino group of Lys. The resulting resin was washed three times each with the following solvents: 0.5% (v/v) DIPEA in DCM, 0.02 M sodium diethyldithiocarbamate in DMF and DMF. Then, Fmoc-Amino acids of ubiquitin sequence were elongated. Note: To suppress the formation of aspartimide byproducts during solid-phase peptide synthesis, Fmoc-Gly(DMB)-OH was installed at the Gly53 residue. The peptidyl resin was washed with DMF×3, DCM×3, MeOH×3, Et_2O×3, and dried under reduced pressure. Dried resin was cleaved using a TFA cocktail (TFA/*m*-cresol/Thioanisole/3,6-Dioxa-1,8-Octanedithiol/H_2O (85/5/5/2.5/2.5, (v/v))) at room temperature. After 2 h, cold Et_2O was added to the reaction mixture to give a precipitate. The formed precipitate was collected by centrifugation and thoroughly washed with Et_2O to afford crude peptide. The crude peptide was purified by reversed-phase HPLC (25-45% solvent B over 90 min, 0.1% TFA, cosmosil 5C_{18}-AR-II 20×250 mm column) followed by lyophilization to yield peptide **8** (1.05 μmol, 2% yield). Analytical HPLC, t_R = 16.8 min (25-35% solvent B over 20 min); HRMS (ESI), Calcd for $C_{408}H_{657}N_{110}O_{124}S_2$ $[M+H]^+$ 9153, found 9153.

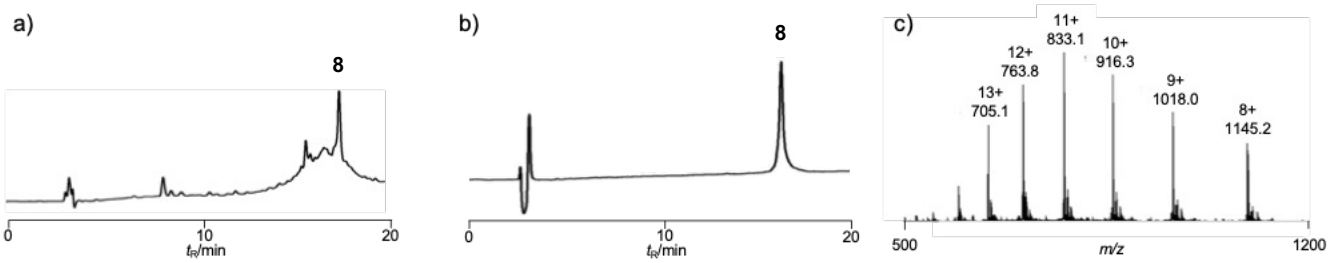

Figure S3: a) HPLC trace of crude material of **8**. b) HPLC trace of purified **8**. c) ESI-MS spectrum of purified **8**.

3. Fluorescence polarization (FP) measurements

We examined the stability of the (*E*)-methylalkene-type Ub mimic (**4**) and (*Z*)-chloroalkene-type Ub mimic (**7**) to enzymatic hydrolysis through the fluorescence polarization (FP) technique. The solution of 5-FAM-labeled Ub derivatives (50 µL, 1 mg/mL in PBS each) were added on a 96-well plate. After addition of a buffer (39 µL, 50 mM Tris, 50 mM NaCl, 2 mM dithiothreitol (pH 8.0)), Usp2cc (11 µL, 0.9 mg/mL in 50 mM Tris/HCl (pH7.5), 100 mM NaCl,10% Glycerol) was added to the well. The resulting plate was immediately set to a microplate reader (Infinite 200 PRO, Tecan, Switzerland) equipped with 485 nm excitation and 535 nm emission filters, and the FP measurements were carried out every 1 min for 60 min at 37 °C.

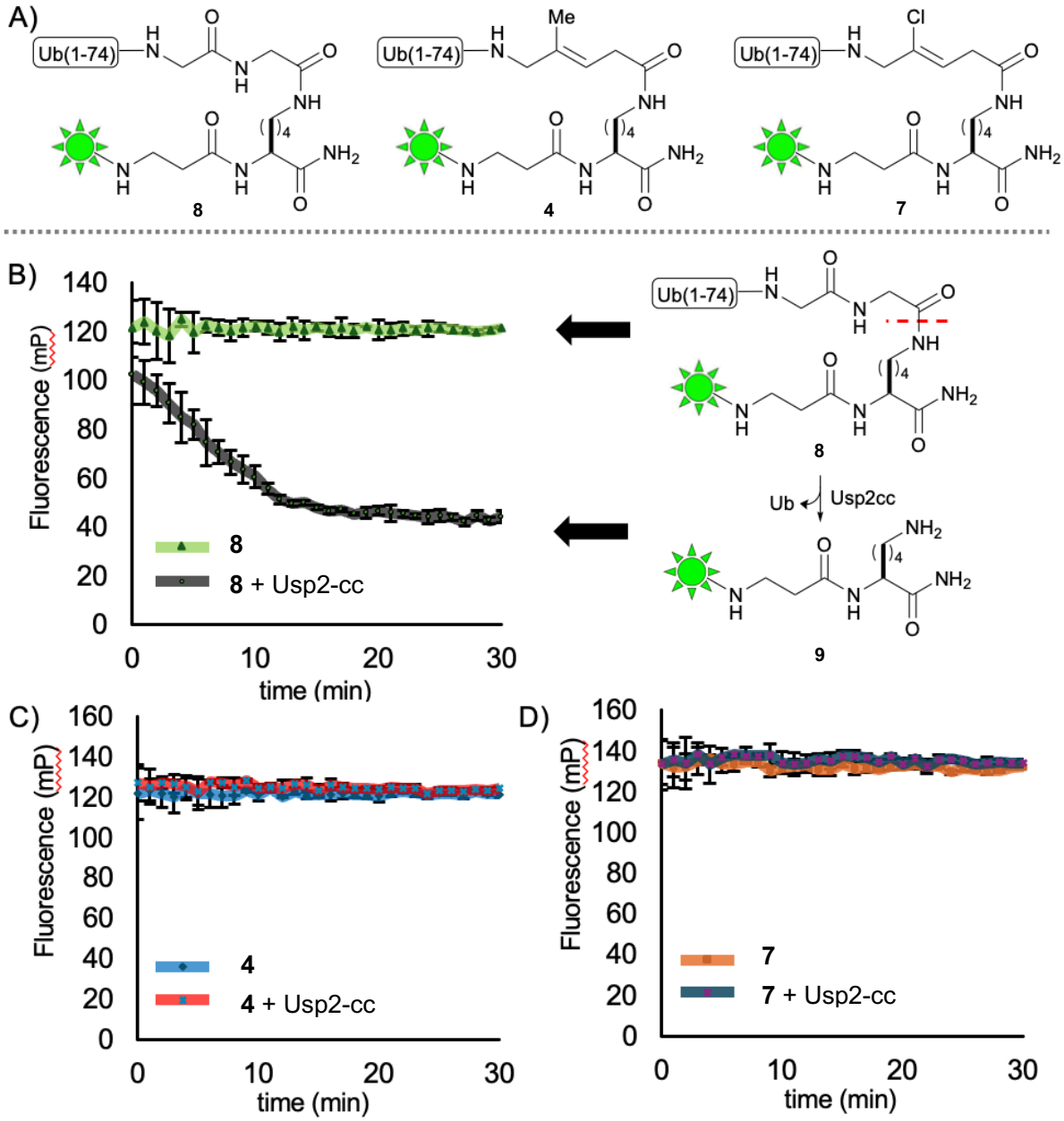

Figure S4 A) Native type Ub **8**, Methylalkene-type Ub **4** and Chloroalkene-type Ub **7**. B) Reaction time course of FP assays with **8** and alkene mimics, C) **4**, and D) **7**.

SDS-PAGE

Proteins denatured in 1× NuPAGE LDS sample buffer (Thermo Fisher Scientific) were heated at 70 ºC for 10 min and, then separated by SDS-PAGE on 4–12% NuPAGE Bis-Tris gels (Invitrogen) with NuPAGE™ MES SDS Running buffer at 150 V for 45 min. The gels were stained with Coomassie Brilliant Blue (CBB).

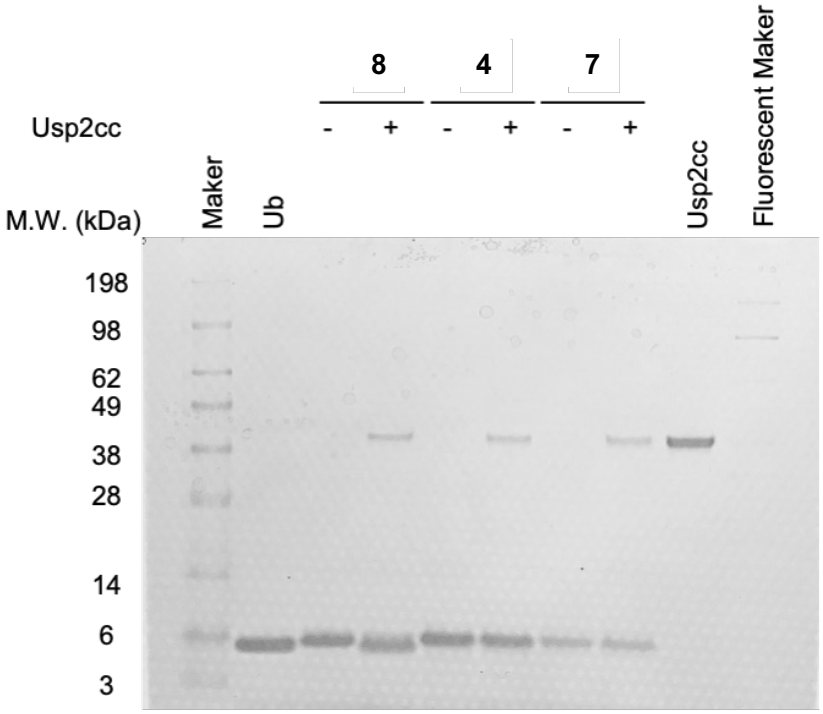

Figure S5 Hydrolysis of Native type Ub **8**, Methylalkene-type Ub **4** and Chloroalkene-type Ub **7** using Usp2cc were analyzed by SDS-PAGE.