Supporting Information

Alkyne-tag SERS screening and identification of small-molecule-binding sites in protein

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Table of Contents:

Supplementary Figures

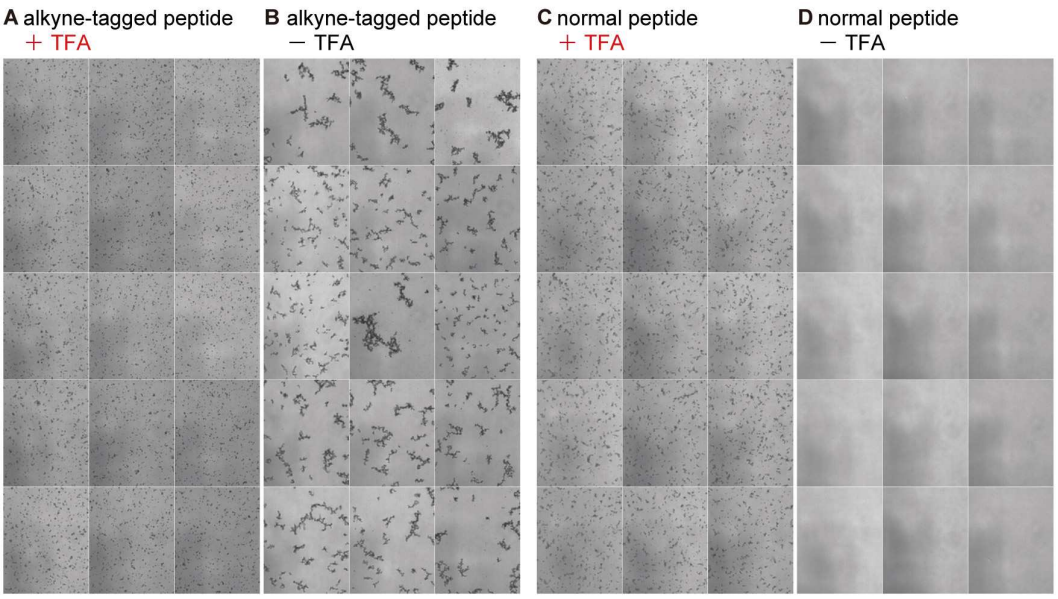


Figure S1. Bright-field images of silver nanoparticles in the presence of (*A*) alt-pept with TFA (*B*) alt-pept without TFA (*C*) pept with TFA and (*D*) pept without TFA (n = 15), taken at 15 different wells.

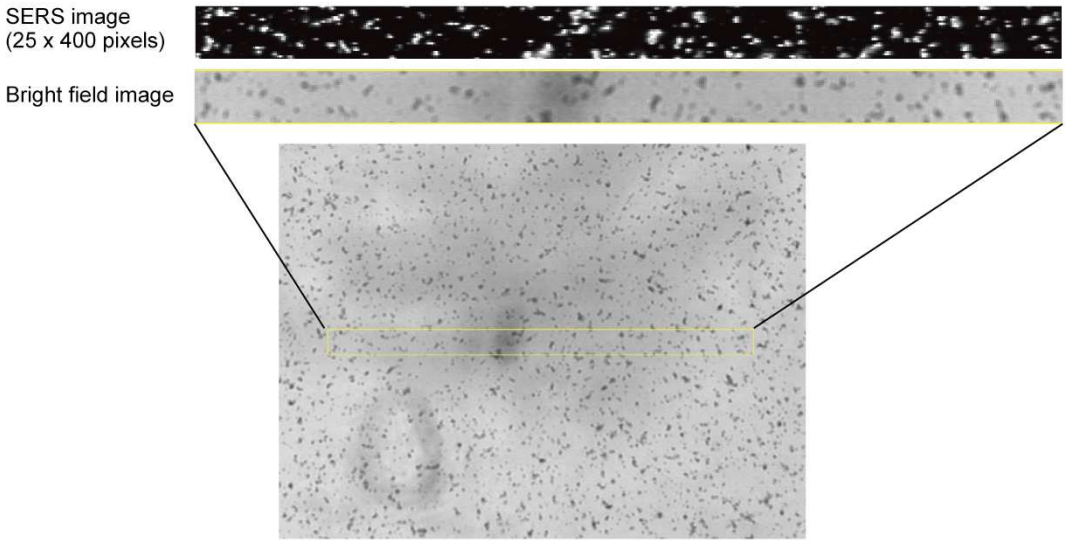


Figure S2. Bright-field and SERS images of 10 pmol alt-pept in TFA-containing solution mixed with silver nanoparticles. The 532 nm excitation laser beam was focused as a line by the 0.75 NA dry objective lens. Exposure time was 1 sec/line for imaging (25 lines, 25 x 400 pixels). The SERS image was reconstructed from the alkyne peak intensity distribution at 1958 cm^{-1}.

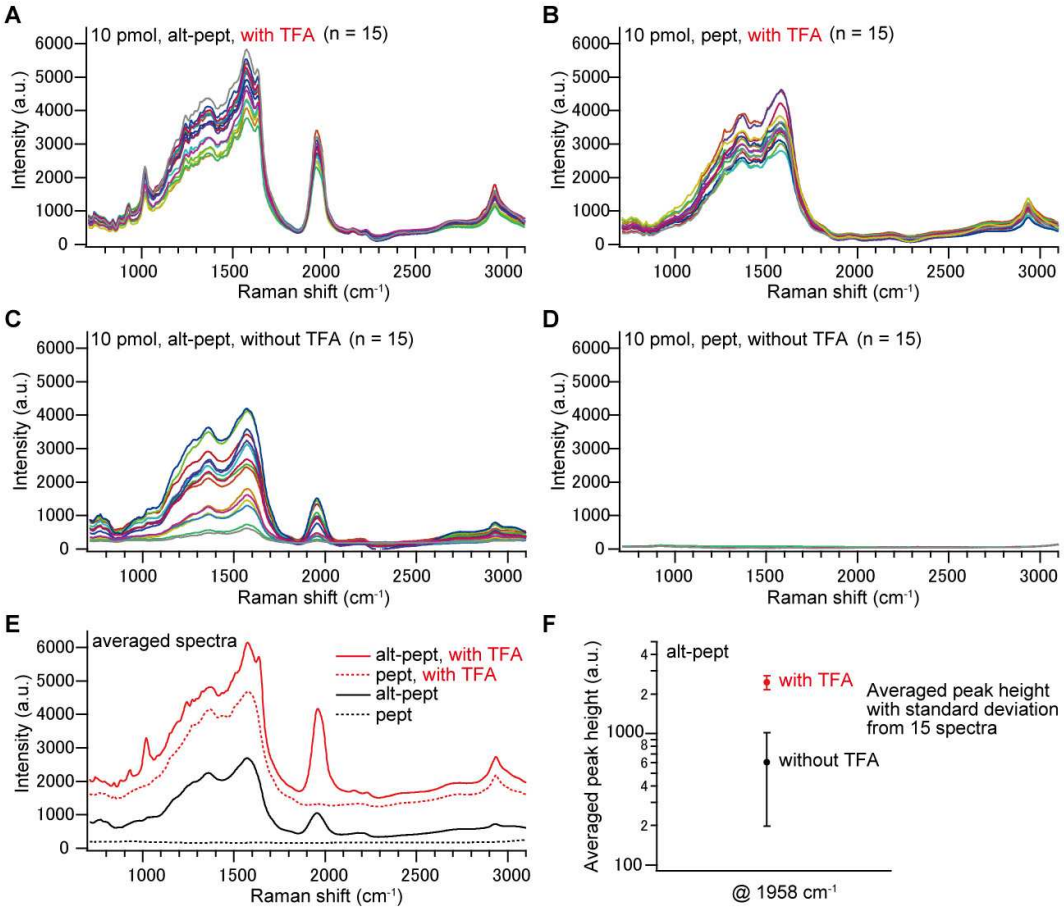


Figure S3. SERS analysis with single-line exposure of 10 pmol peptides with/without alkyne tag, and with/without TFA mixed with silver nanoparticles. SERS measurement was performed with a single laser line exposure at an exposure time of 3 sec (average spectrum of 400 points). (*A*) SERS spectra of 10 pmol alt-pept in TFA-containing solution (n = 15, measured at different wells). (*B*) SERS spectra of 10 pmol pept without alkyne-tag in TFA-containing solution (n = 15). (*C*) SERS spectra of 10 pmol alt-pept without TFA (n = 15). (*D*) SERS spectra of 10 pmol pept without TFA (n = 15). (*E*) Averaged spectra in Figure S3 *A* to *D*. (*F*) Averaged peak heights and their standard deviation for the averaged SERS spectra of alt-pept with and without TFA.

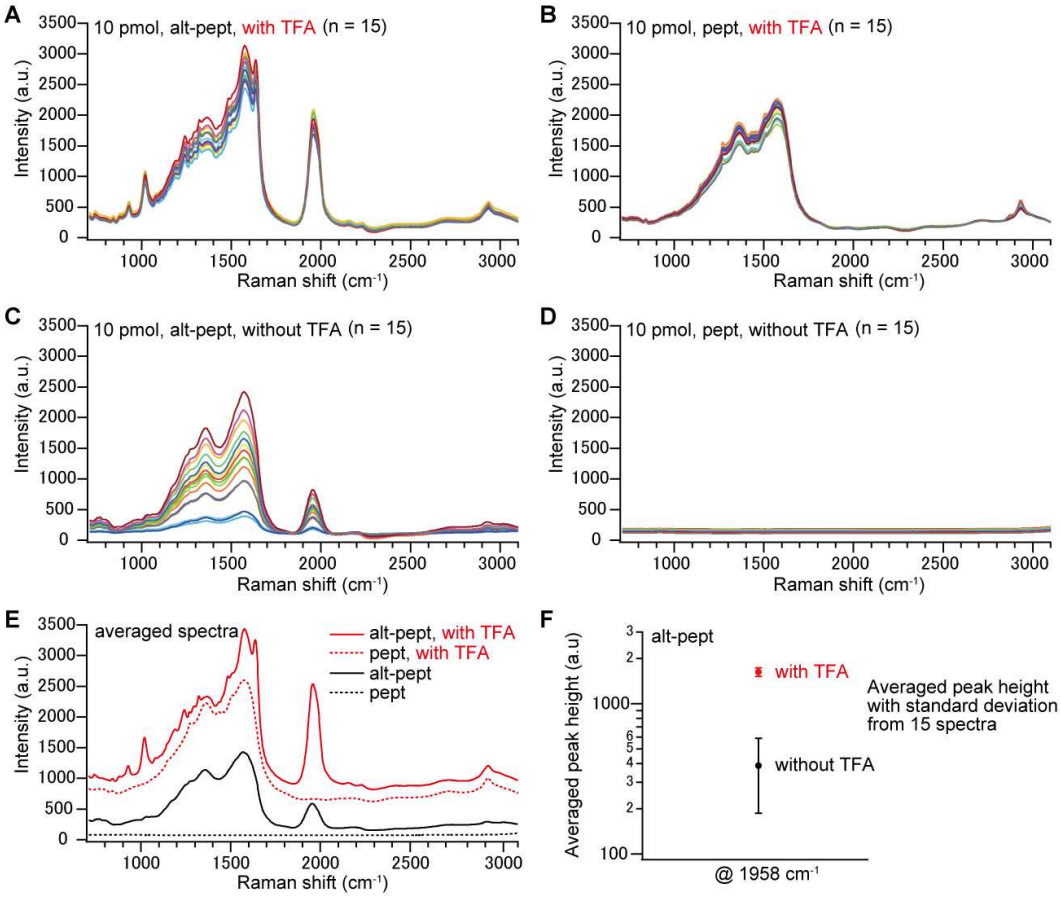


Figure S4. SERS analysis with line imaging of 10 pmol peptides with/without alkyne tag, and with/without TFA mixed with silver nanoparticles. SERS measurement was performed using line imaging at an exposure time of 1 sec with 25 lines (average spectrum from 10,000 points). (*A*) SERS spectra of 10 pmol alt-pept in TFA-containing solution (n = 15, measured at different wells). (*B*) SERS spectra of 10 pmol pept in TFA-containing solution (n = 15). (*C*) SERS spectra of 10 pmol alt-pept without TFA (n = 15). (*D*) SERS spectra of 10 pmol pept without TFA (n = 15). (*E*) Averaged spectra in Figure S4 *A* to *D*. (*F*) Averaged peak heights and their standard deviation for the averaged SERS spectra of alt-pept with and without TFA.

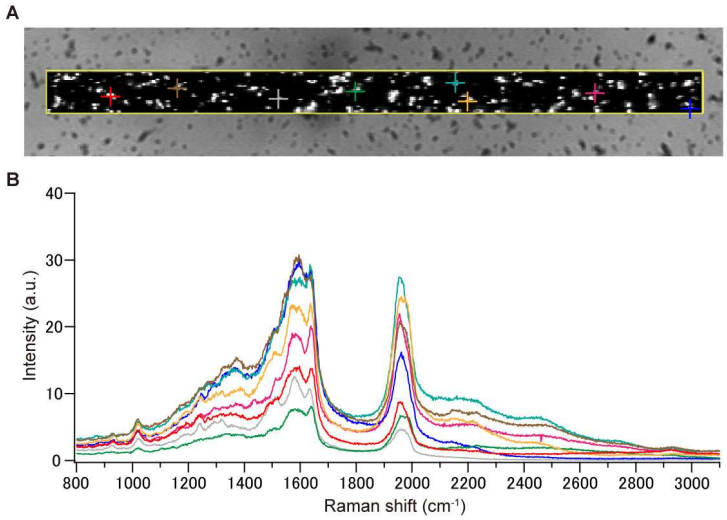


Figure S5. (*A*) SERS image of 10 pmol of alt-pept aggregated with silver nanoparticles, overlaid on the bright-field image. (*B*) SERS spectra obtained from different aggregates indicated in the SERS image. The color of each SERS spectrum corresponds to that of the cross in the SERS image.

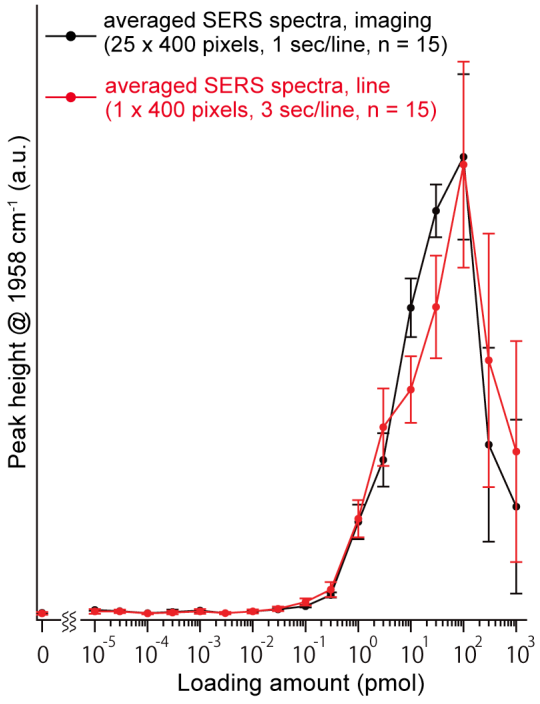


Figure S6. Relationship between SERS peak intensity at 1958 cm^{-1} and the loading amount of alt-pept with silver nanoparticles in the presence of TFA. SERS spectra are the averages of single line exposure and line imaging (n = 15).

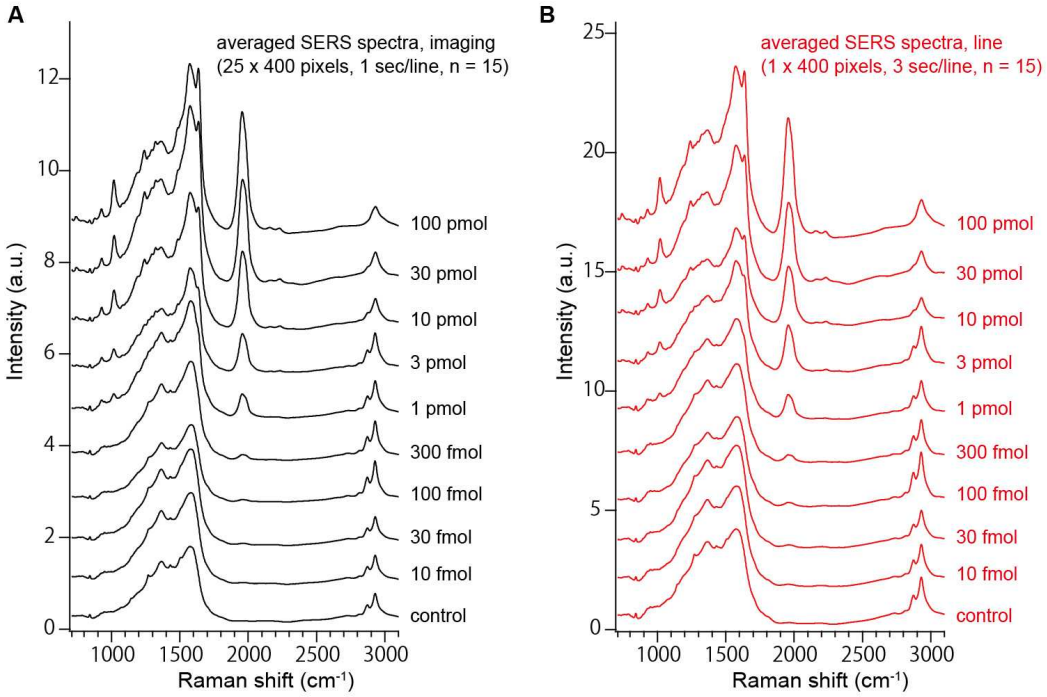


Figure S7. Averaged SERS spectra of alt-pept with silver nanoparticles in the presence of TFA with various loading amounts of peptide. SERS spectra are the averages of (*A*) line imaging and (*B*) single line exposure (n = 15).

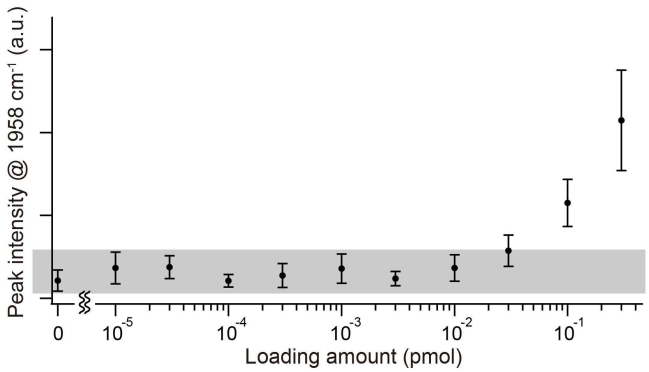


Figure S8. Enlarged view of the relationship between SERS peak intensity at 1958 cm^{-1} and the loading amount of alt-pept with silver nanoparticles in the presence of TFA below 300 fmol of alt-pept. SERS spectra are the averages of single line exposure (n = 15).

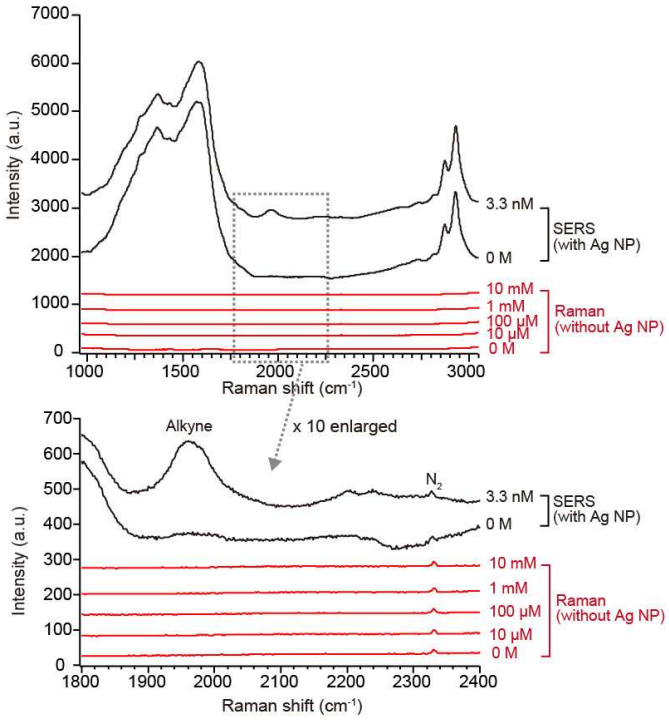


Figure S9. Red lines: Raman spectra of alt-pept in solution at different concentration (0 M and 10 µM~10 mM) without silver nanoparticles. Black lines: Raman spectra of alt-pept in solution at 0 M and 3.3 nM with silver nanoparticles. Both Raman and SERS spectra were obtained under same measurement conditions.

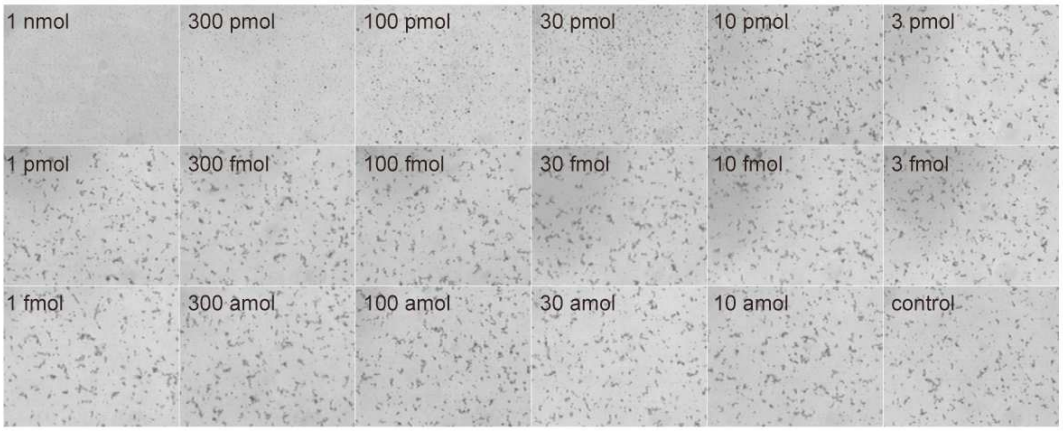


Figure S10. Bright-field images of silver nanoparticle aggregates in the presence of TFA with different loading amounts of alt-pept from 10 amol to 1 nmol and 0 mol (control). Each picture is a representative image from 15 samples.

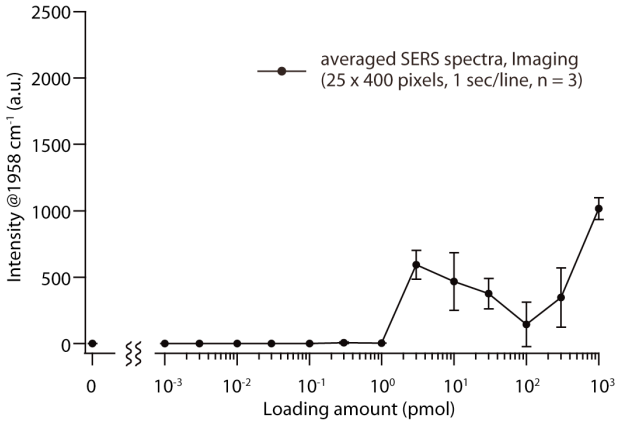


Figure S11. Relationship between SERS peak intensity at 1958 cm^{-1} and the loading amount of alt-pept with silver nanoparticles in the absence of TFA. SERS spectra were obtained as the average of line imaging (n = 3).

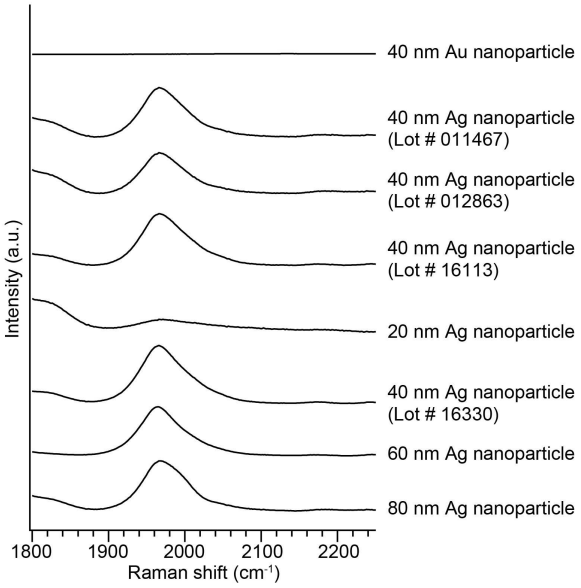


Figure S12. SERS spectra of alt-pept with metal nanoparticles in the presence of TFA with various conditions, using 20 nm, 40 nm, 60 nm, and 80 nm silver nanoparticles; 4 different batches of 40 nm silver nanoparticles; and 40 nm gold nanoparticles.

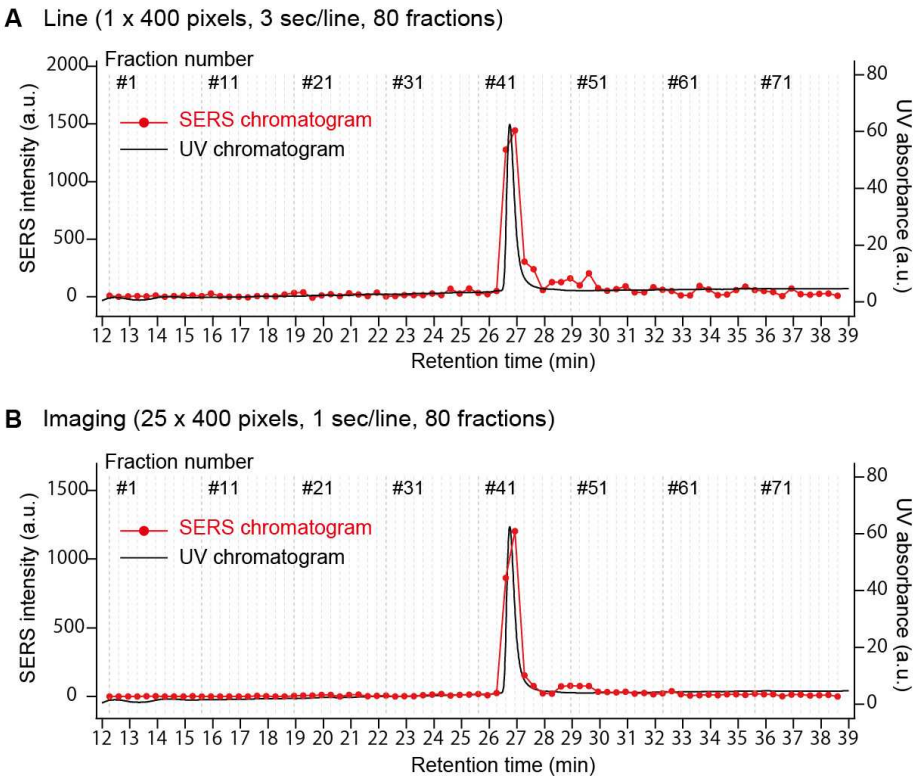


Figure S13. SERS chromatogram of 10 pmol alt-pept prepared by HPLC with a fraction collector, obtained with (*A*) single line exposure (3 sec, 1 line/well) and (*B*) line imaging (1 sec, 25 lines/well). SERS intensity was calculated as alkyne peak height at 1958 cm^{-1}. UV absorbance was measured at 215 nm.

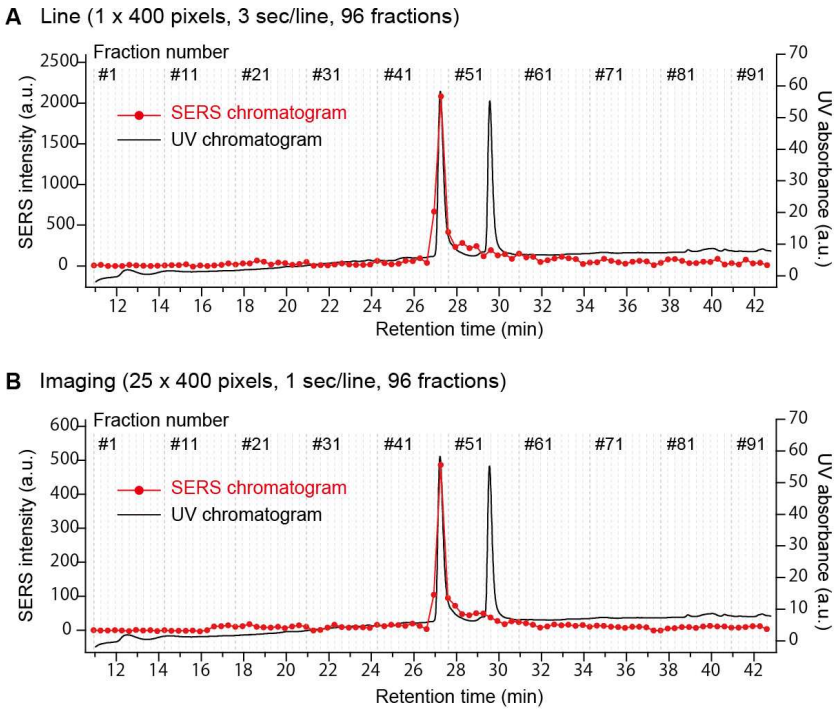


Figure S14. SERS chromatogram of a mixture of 10 pmol alt-pept and pept prepared by HPLC with a fraction collector, obtained with (*A*) single line exposure (3 sec, 1 line/well) and (*B*) line imaging (1 sec, 25 lines/well). SERS intensity was calculated as peak height of alkyne at 1958 cm^{-1}. UV absorbance was measured at 215 nm.

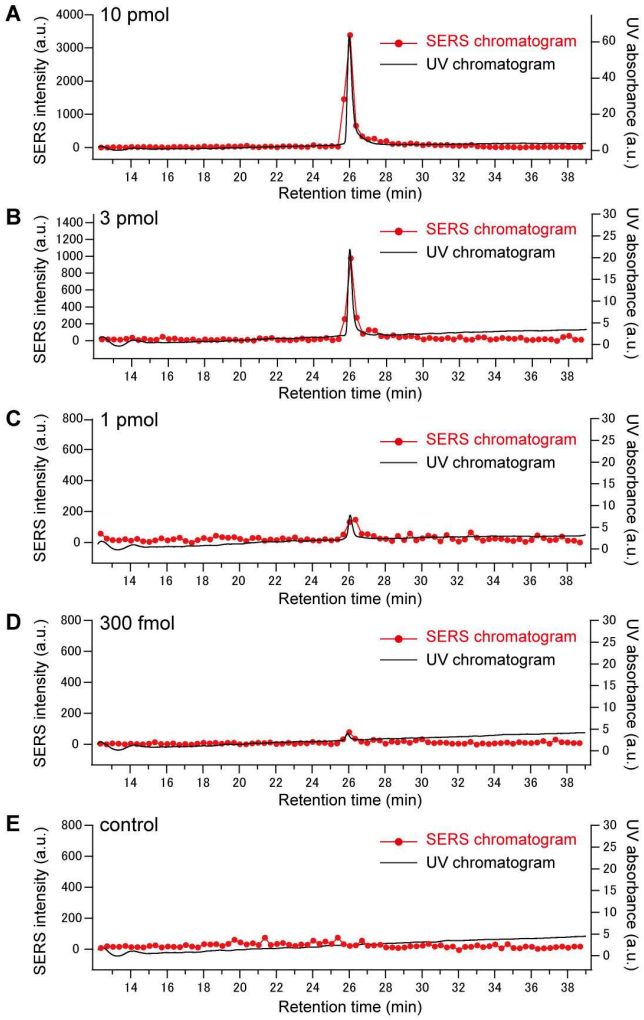


Figure S15. SERS chromatograms of alt-pept at various loading amounts: (*A*) 10 pmol (*B*) 3 pmol, (*C*) 1 pmol, (*D*) 300 fmol and (*E*) 0 mol (control). Each sample was prepared by HPLC with a fraction collector. SERS intensity was calculated as alkyne peak height at 1958 cm^{-1} with single line exposure (3 sec, 1 line/well). UV absorbance was measured at 215 nm.

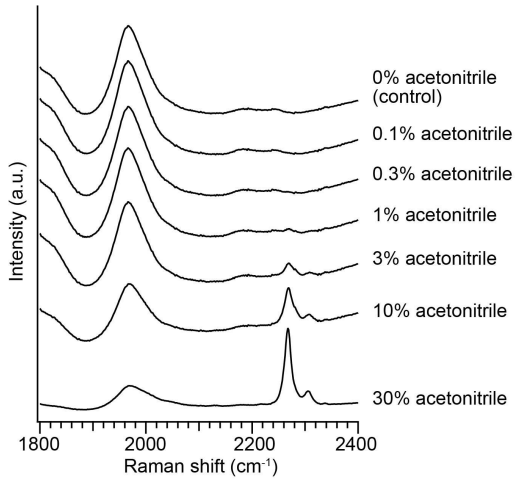


Figure S16. SERS spectra of alt-pept with silver nanoparticles in the presence of TFA with different concentrations of acetonitrile in solution.

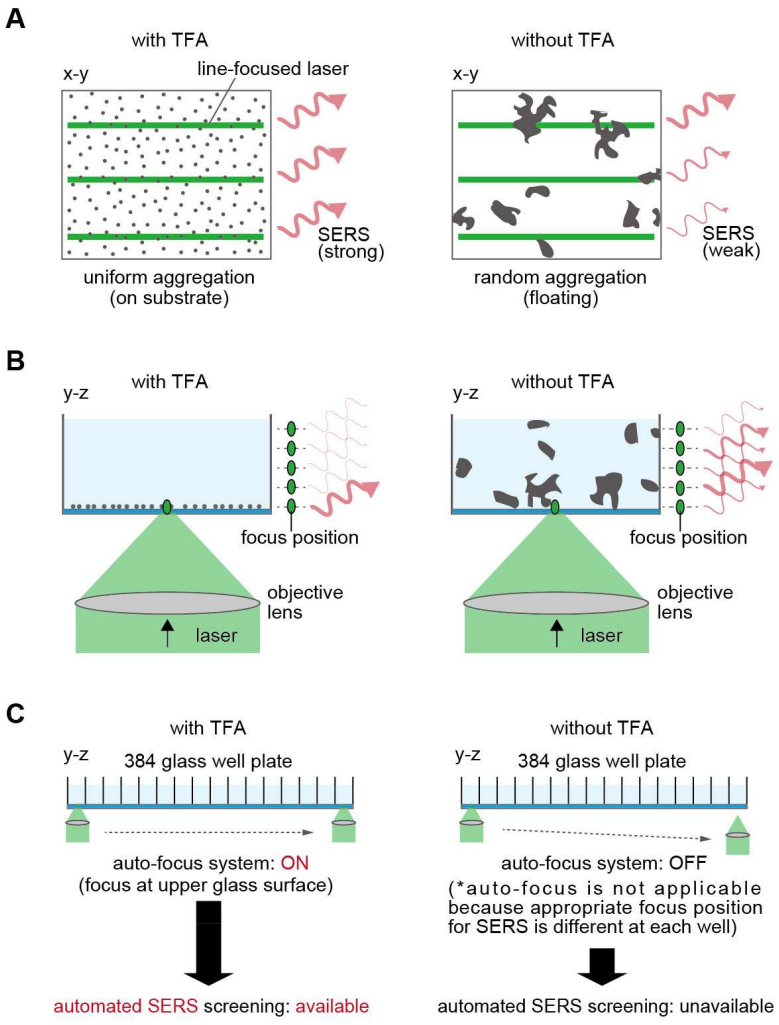


Figure S17. Automated screening using ATRaS technology. (*A*) Automation is feasible due to the uniform formation of nanoparticle aggregates on the substrate in the presence of TFA, which means that sample position search in each well, to find hot spot that gives strong SERS signal, is not necessary in moving from well to well. (*B*) Localized aggregate formation at the surface of the glass substrate, which allows us to avoid focus position search to find SERS hot spot. (*C*) Localized aggregation allowed us to use an auto-focus system to keep the Raman excitation conditions the same at each well. Furthermore, the strong Raman signal amplification shortened the exposure time for SERS detection to 3 seconds per well.

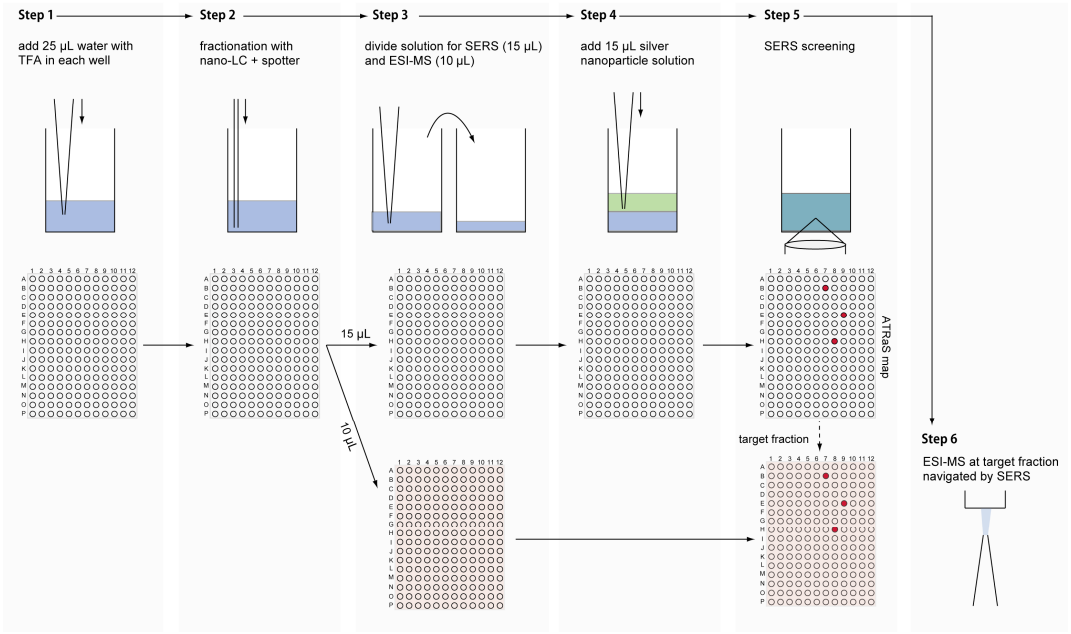


Figure S18. Schematic illustration of experimental procedures for ATRaS and subsequent ESI-MS analysis.

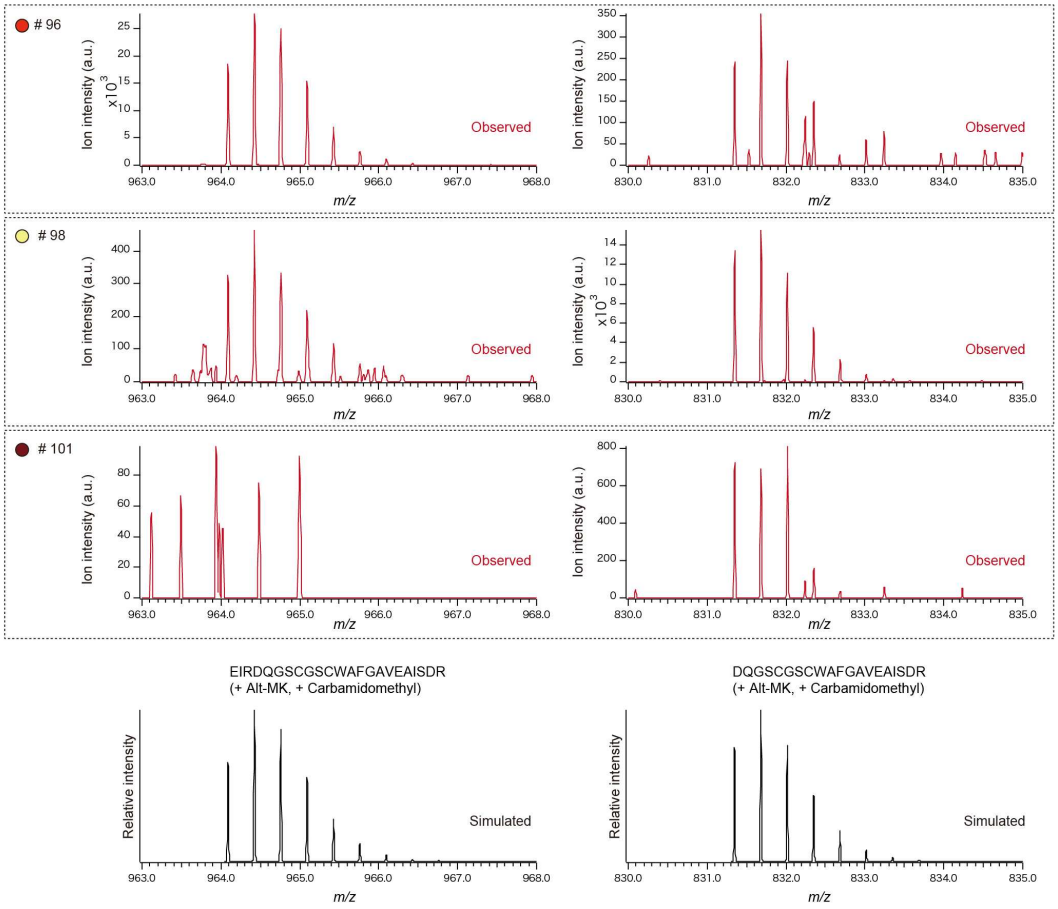


Figure S19. ESI-MS spectra obtained at fraction number 96 and 98 in Figure 6f. Simulated MS spectra of $[M+3H]^{3+}$ of ^{19}EIRDQGSCGSCWAFGAVEAISDR^{41} and ^{22}DQGSCGSCWAFGAVEAISDR^{41}, which are labeled with Alt-AOMK, are also displayed. Strong signal of $[M+3H]^{3+}$ of ^{19}EIRDQGSCGSCWAFGAVEAISDR^{41} that is labeled with Alt-AOMK, was observed at fraction #96. Strong signal of $[M+3H]^{3+}$ of ^{19}DQGSCGSCWAFGAVEAISDR^{41} that is labeled with Alt-AOMK, was observed at fraction #98, as summarized in Table 1. ESI-MS spectra obtained at fraction number 101, where weak SERS signal was obtained, is also displayed. Although the signal is weak, $[M+3H]^{3+}$ of ^{19}DQGSCGSCWAFGAVEAISDR^{41} that is labeled with Alt-AOMK, was observed at fraction #101.

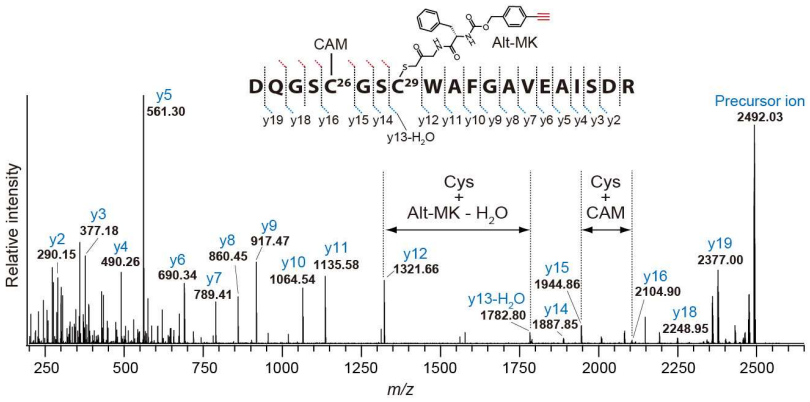


Figure S20. MALDI-Orbitrap MS/MS spectrum of the ion peak derived from DQGSCGSCWAFGAVEAISDR that is labeled with Alt-AOMK. Ion at *m/z* 2492.03 is selected as precursor ion. Fragmentation pattern represents that C^{29} was labeled with Alt-MK, while C^{26} was modified with carbamidomethyl group. CAM is the abbreviation for carbamidomethylation.

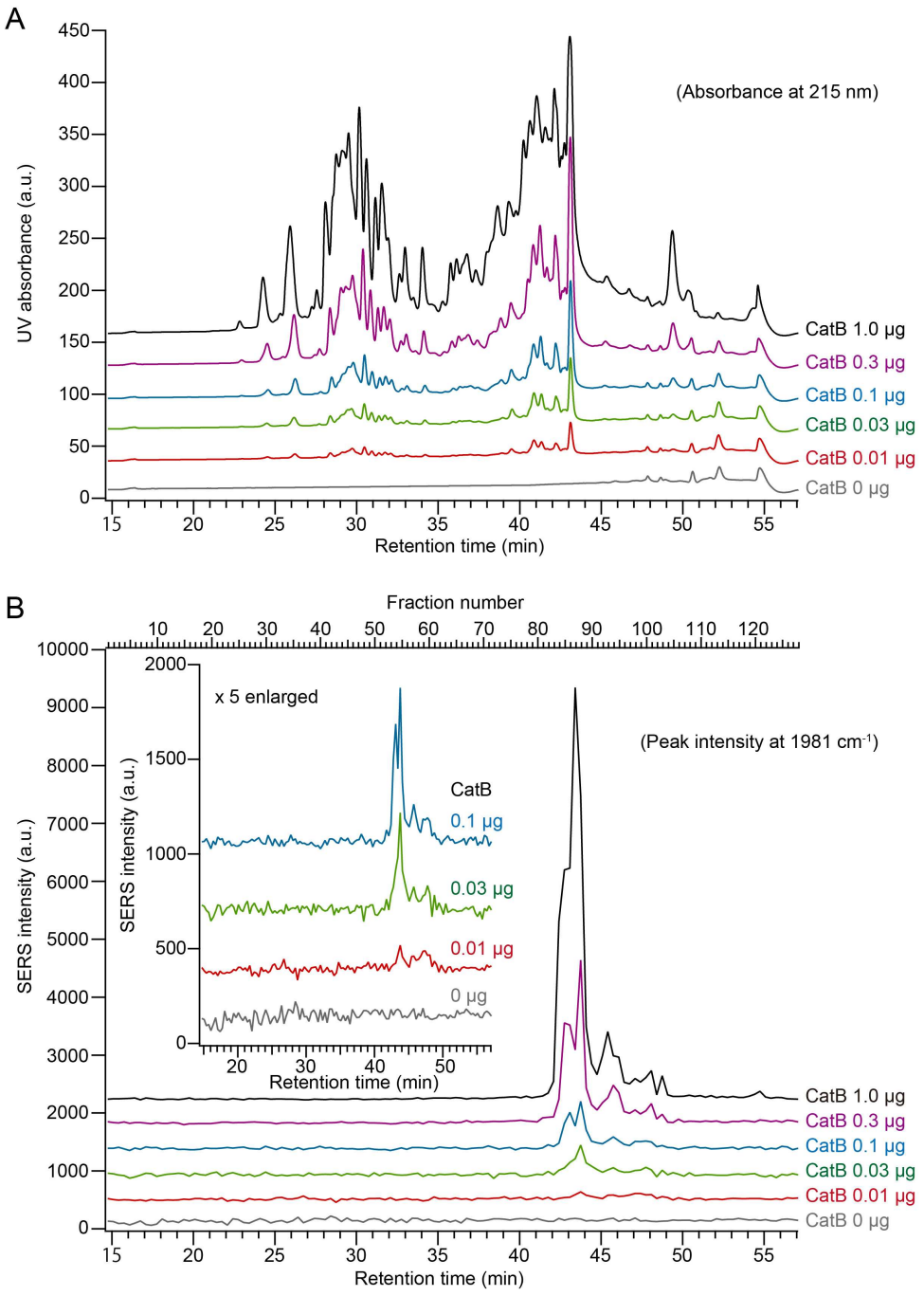


Figure S21. (A) UV and (B) SERS chromatogram of trypsin-digested CatB treated with Alt-AOMK. Loading amount of CatB was changed from 1.0 μg to 0.01 μg and 0 μg (control). SERS measurement was performed with single line exposure (5 sec, 1 line/well). SERS intensity was calculated as alkyne peak height at 1981 cm^{-1}. UV absorbance was measured at 215 nm.

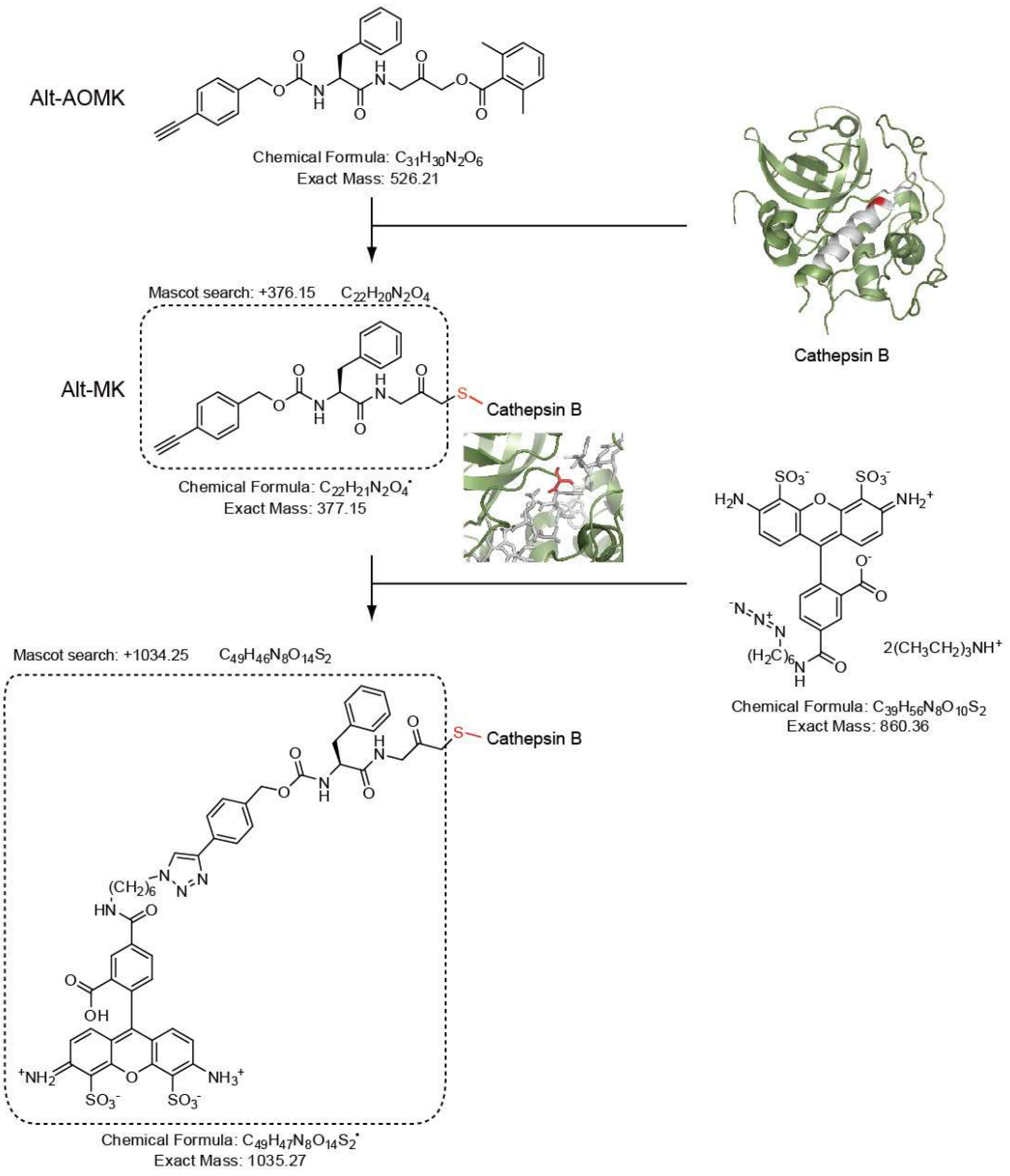


Figure S22. Expected reaction and mass shifts between CatB, Alt-AOMK and Alexa Fluor® 488 Azide (1, 2).

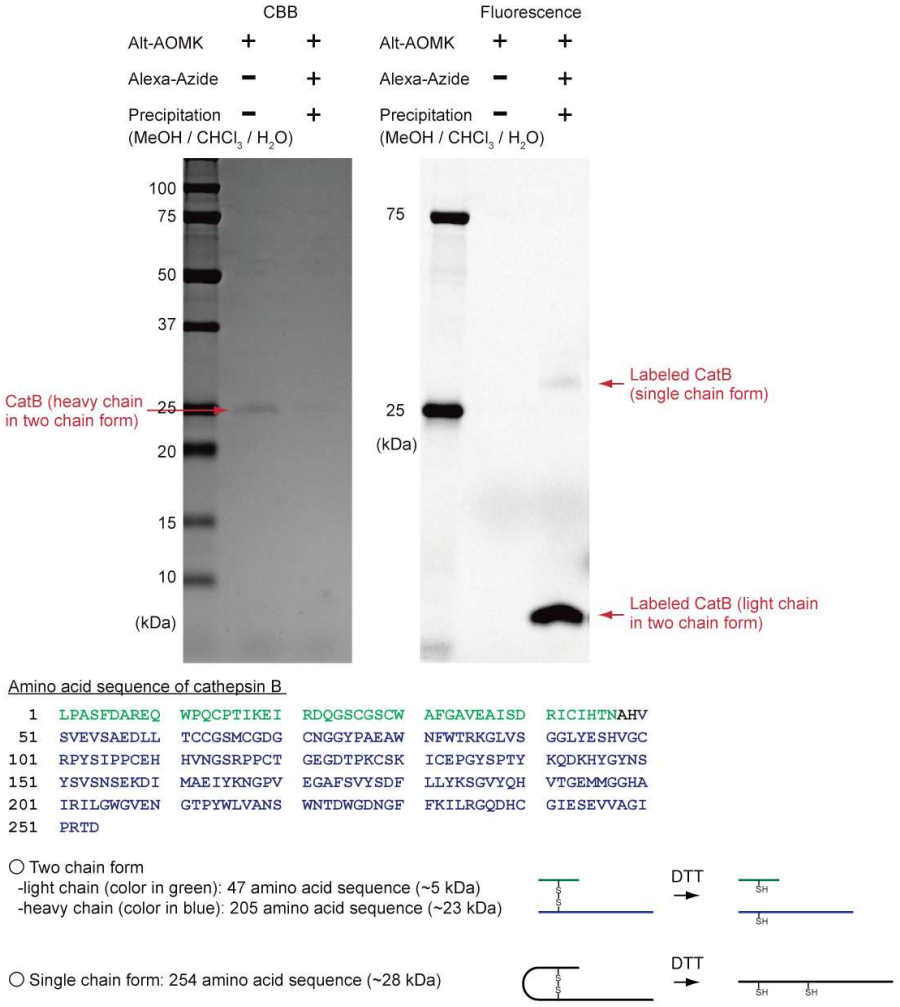


Figure S23. SDS-PAGE of Alt-AOMK-labeled CatB with and without click reaction using Alexa Fluor® 488 Azide, and subsequent precipitation using MeOH/$CHCl_3$/H_2O. Click reaction was performed with Click-iT® protein reaction buffer kit. Cathepsin B has two mature forms, a single-chain form, and a two-chain form composed of light chain (~5 kDa) and heavy chain (~23 kDa). In this sample, the two-chain form was dominant, and the heavy chain was observed as a major band in the CBB-stained gel. After click reaction, the intensity of the heavy chain band became lower, indicating loss of sample. On the other hand, in the fluorescence image, the light chain containing the catalytic cysteine residue was detected as a major band, implying that catalytic cysteine was labeled with Alt-AOMK and Alexa Fluor® 488 Azide. A small amount of single-chain form (~28 kDa) was also detected as a minor band in fluorescence imaging.

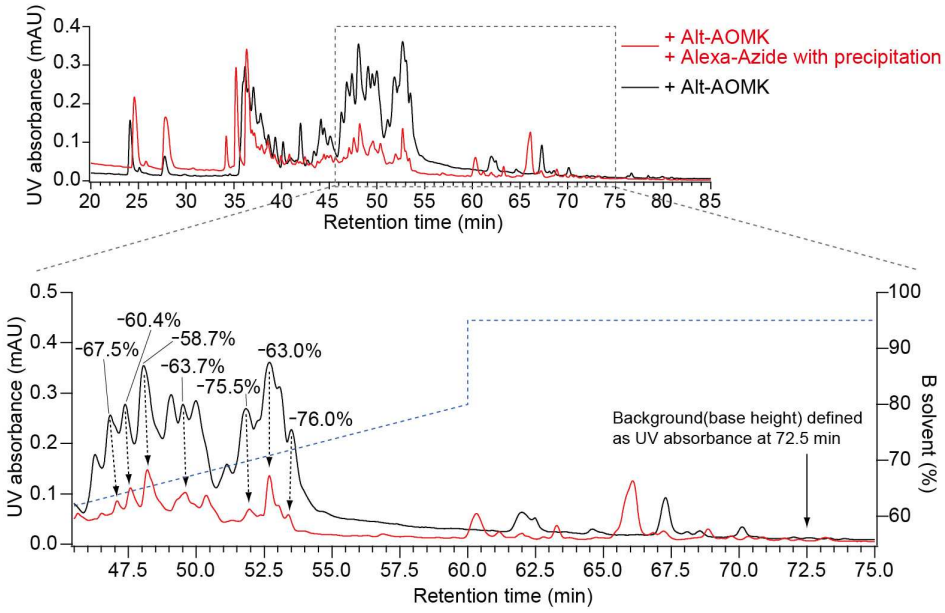


Figure S24. UV chromatogram of trypsin-digested CatB treated with Alt-AOMK (black line) and that of trypsin-digested CatB treated with both Alt-AOMK and Alexa Fluor® 488 Azide (red line). The values in enlarged chromatograms show sample loss due to click reaction and precipitation, calculated from the peak height of each chromatogram.

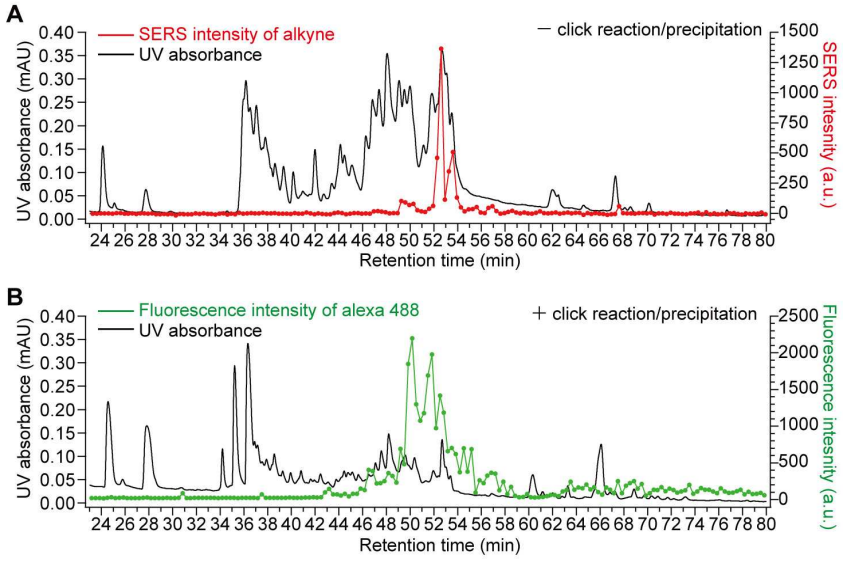


Figure S25. (*A*) UV and SERS chromatograms of trypsin-digested CatB treated with Alt-AOMK (*B*) UV and fluorescence chromatograms of trypsin-digested CatB treated with Alt-AOMK and Alexa Fluor® 488 Azide.

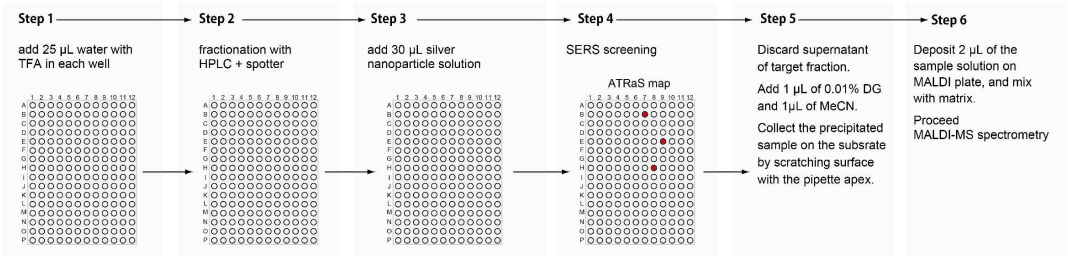


Figure S26. Schematic illustration of experimental procedures for ATRaS and subsequent MALDI-TOF MS analysis.

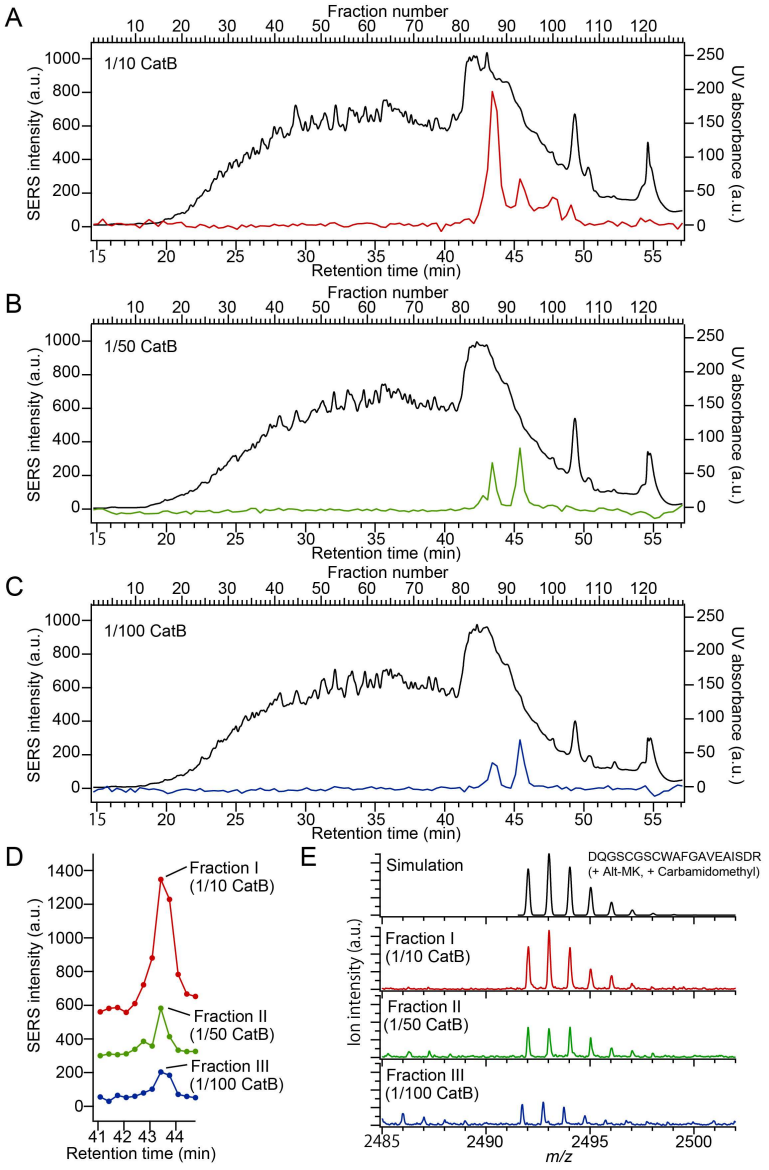


Figure S27. UV and SERS chromatogram of tryptic digest of the Alt-AOMK-treated HL60 cell lysate mixed with CatB at (A) a tenth part, (B) fiftieth part, and (C) a hundredth part of total proteins. The protein mixture was treated with Alt-AOMK, and digested by trypsin. Loading amount of the total protein volume was 2.5 μg for each experiment. SERS measurement was performed with single line exposure (5 sec, 1 line/well). SERS intensity was calculated as alkyne peak height at 1981 cm^{-1}. UV absorbance was measured at 215 nm. (D) Enlarged view of the peak around fraction 87 in SERS chromatograms of Fig. S27 A to C. (E) MALDI-TOF MS spectra measured at target fraction indicated in Fig. S27D. Simulated and observed $[M+H]^+$ of Alt-MK- and CAM-modified ^{22}DQGSCGSCWAFGAVEAISDR^{41} peptide.

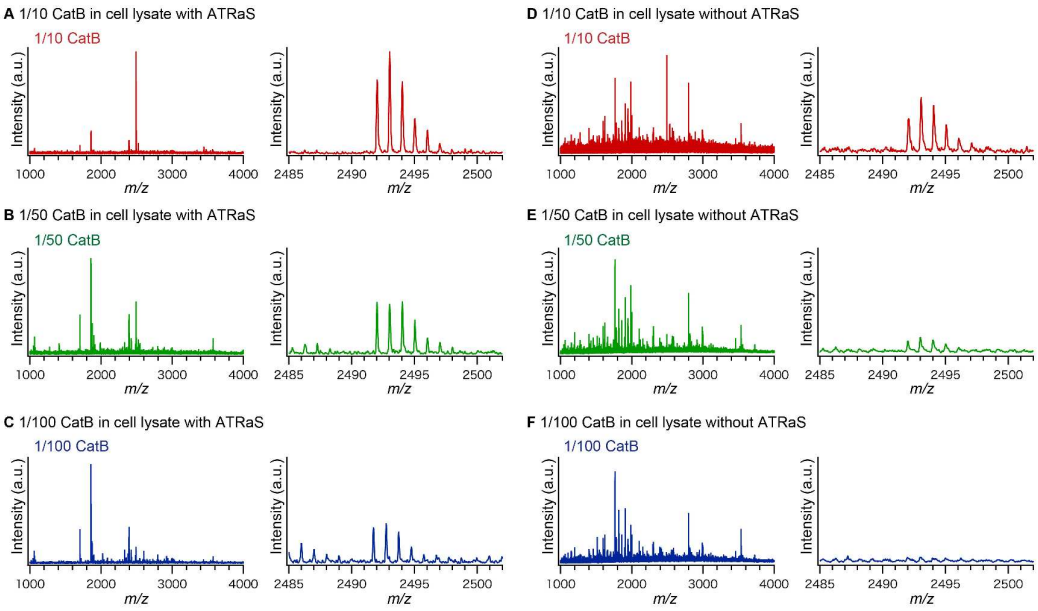


Figure S28. LC-SERS-MALDI-TOF MS analysis of tryptic digest of the Alt-AOMK-treated HL60 cell lysate mixed with CatB at (A) a tenth part, (B) fiftieth part, and (C) a hundredth part of total proteins, and direct MALDI-TOF MS analysis of the tryptic digest of the Alt-AOMK-treated HL60 cell lysate mixed with CatB at (D) a tenth part, (E) fiftieth part, and (F) a hundredth part of total proteins. Loading amount of the total protein volume was the same between LC-SERS-MALDI-TOF MS (2.5 μg was loaded in HPLC) and direct MALDI-TOF MS (2.5 μg was loaded on MALDI plate) measurement. Ions corresponding $[M+H]^+$ of Alt-MK- and CAM-modified ^{22}DQGSCGSCWAFGAVEAISDR^{41} were observed for (A) ~ (E).

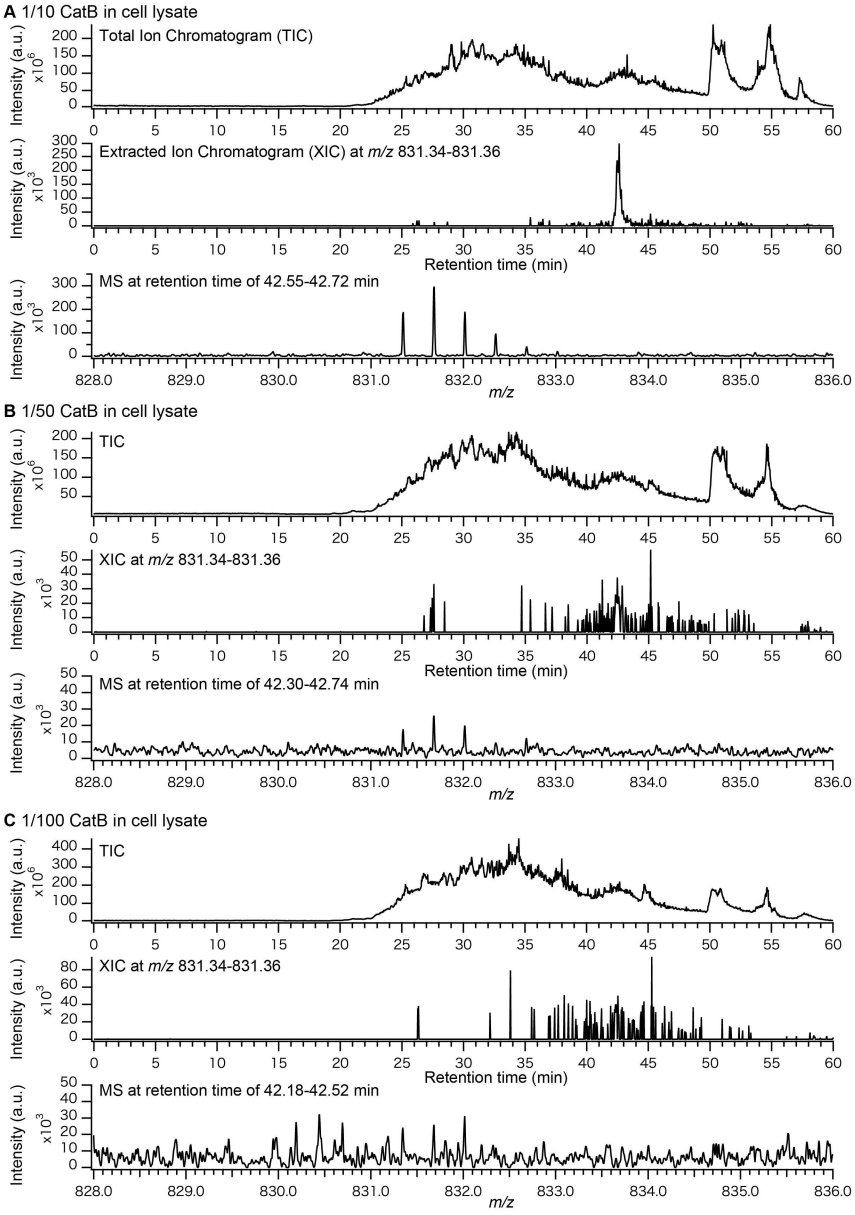


Figure S29. Direct LC-MS analysis of tryptic digest of the Alt-AOMK-treated HL60 cell lysate mixed with CatB at (A) a tenth part, (B) fiftieth part, and (C) a hundredth part of total proteins. The protein mixture treated with Alt-AOMK was digested by trypsin. Loading amount of the total protein volume was approximately 0.2 μg for A and B, and was approximately 0.4 μg for C. The total ion current (TIC) chromatograms, the extracted ion chromatograms (XIC) at *m/z* 831.34-831.36 of $[M+3H]^{3+}$ corresponding to Alt-MK and CAM-modified ^{22}DQGSCGSCWAFGAVEAISDR^{41} peptide, and mass spectra corresponding to the peaks in XIC at retention time of around 42 min are shown.

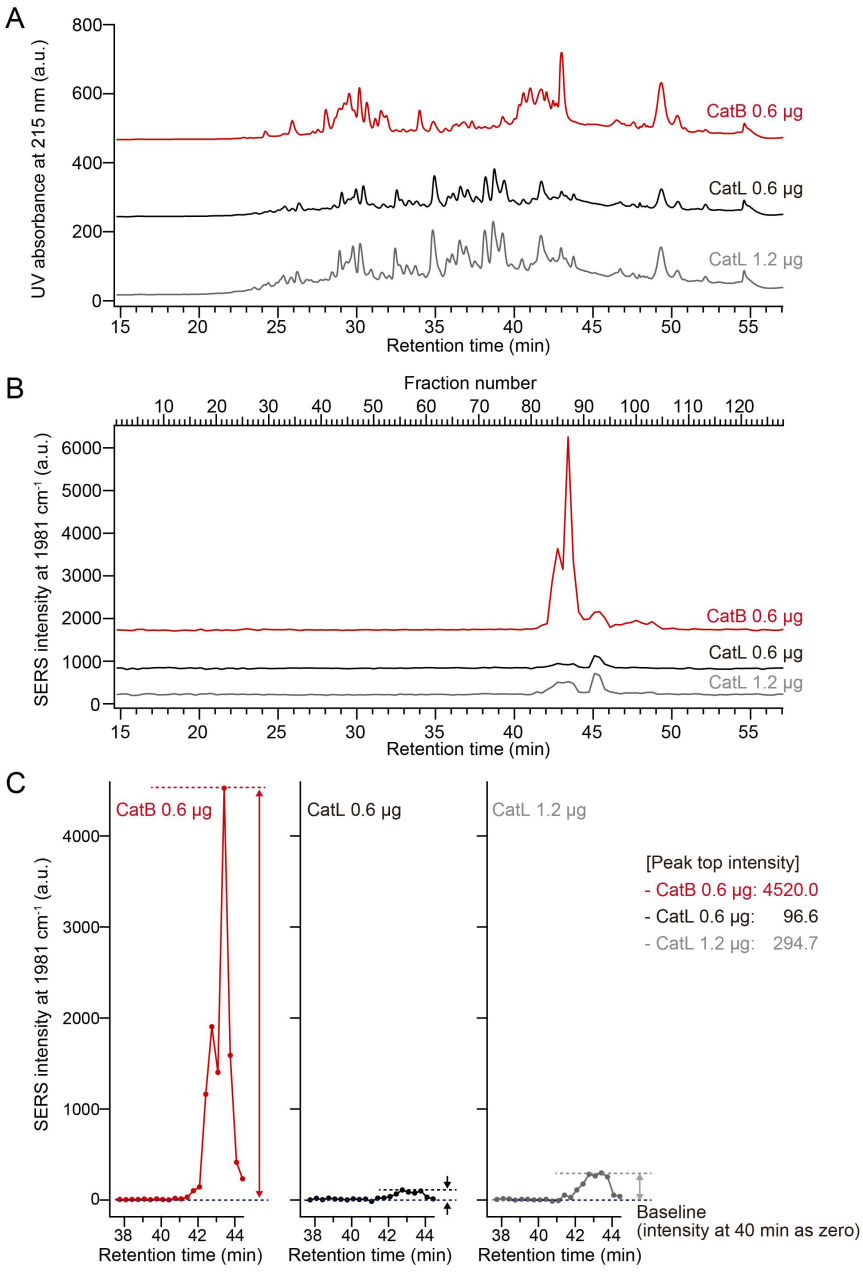


Figure S30. (A) UV and (B) SERS chromatogram of trypsin-digested CatB and CatL treated with Alt-AOMK. Loading amount of CatB is 0.6µg. Loading amount of CatL is 0.6 µg and 1.2 µg. SERS measurement was performed with single line exposure (5 sec, 1 line/well). SERS intensity was calculated as alkyne peak height at 1981 cm^{-1}. UV absorbance was measured at 215 nm. (C) Enlarged view of the peak around the retention time of 43 min in SERS chromatograms of Fig. S30B. Peak top intensity of each sample was calculated by using the bottom intensity at retention time of 40 min.

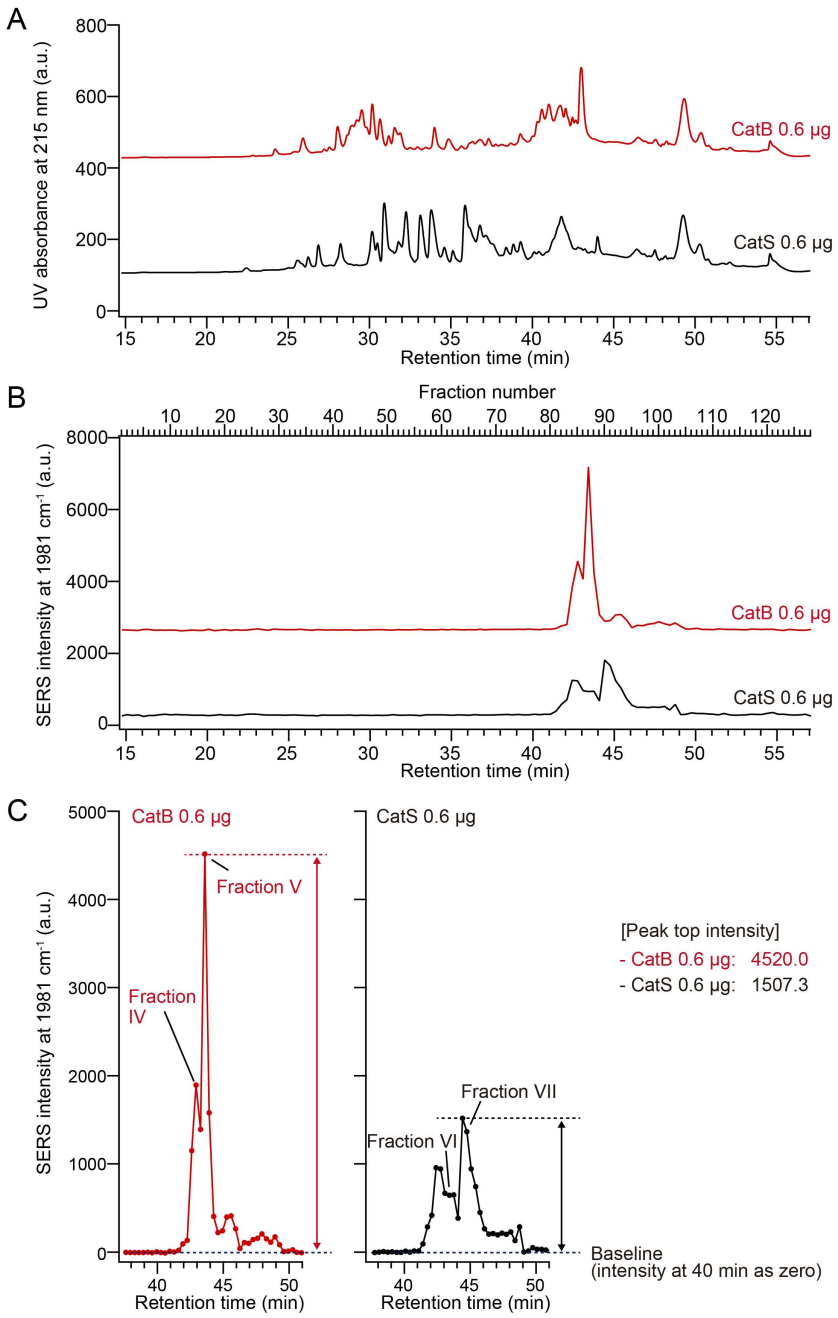


Figure S31. (A) UV and (B) SERS chromatogram of trypsin-digested CatB and CatS treated with Alt-AOMK. Loading amount of both CatB and CatS is 0.6μg. SERS measurement was performed with single line exposure (5 sec, 1 line/well). SERS intensity was calculated as alkyne peak height at 1981 cm^{-1}. UV absorbance was measured at 215 nm. (C) Enlarged view of the major peaks around the retention time of 44 min in SERS chromatograms of Fig. S31B. Peak top intensity of each sample was calculated by using the bottom intensity at retention time of 40 min.

Supplementary Experimental Procedures

Experimental procedures for Raman analysis of alkyne-tagged synthetic peptide in dried condition (Figure 2b, lower panel).

1 μL of 1 mM alt-pept solution (1 nmol) was deposited on the quartz substrate, and the water was allowed to evaporate to form peptide aggregates. The sample was set on the Raman microscope stage. A continuous-wave 532 nm excitation laser was focused as a point by 0.75 NA dry objective lens (CFI Plan Apo λ 40x, Nikon). Laser power was 325 mW, measured after the objective lens. Exposure time was set as 20 sec.

Experimental procedures for Raman analysis of alkyne-tagged synthetic peptide in solution (Figure S9).

Aqueous solution of synthetic peptides EQWPQCPTXK (X = propargyl glycine, alt-pept) at 0 or 10 μM ~ 10 mM concentration was dropped in a glass-bottomed 384-well plate (EZView assay plate, AGC). The 384-well plate was set on the Raman microscope stage (Raman-11, Nanophoton). A continuous-wave 532 nm excitation laser was focused as a line at each well by a 0.75 NA dry objective lens (CFI Plan Apo λ40x, Nikon). Laser power was 240 mW, measured after the objective lens. Exposure time was set as 3 sec/line for single line exposure (1 line, 1 x 400 pix). The Raman spectrum of each well was obtained by averaging the Raman image.

MS/MS analysis of trypsin-digested peptide of Alt-AOMK-labeled CatB (Figure S20).

Trypsin-digested peptide mixture of Alt-AOMK-labeled CatB was fractionated on a Nano Frontier nLC (Hitachi High-Technologies) equipped with a MU701 UV detector. Peptides were separated on an L-column2 ODS (3 μm, 0.1 x 150 mm, CERI) with a monolithic trap column C18-50-150 (50 μm, 150 x 0.05 mm, Hitachi High-Technologies) at flow rate of 250 nL/min. The UV chromatogram was obtained with a UV detector (MU701, GL Science) set at 215 nm. For the analytical column, mobile phase A (distilled water containing 0.1% (v/v) TFA and 2% (v/v) MeCN) and mobile phase B (0.08% (v/v) TFA and 98% (v/v) MeCN) were utilized to prepare a gradient as follows. 0-60 min: 5-80%, 60.01-75 min: 95% B, 75.01-105 min 5% B. The eluate was fractionated at 20 sec intervals (Probot, Dionnex) on a MALDI target (Thermo Scientific, ITOP plate). For mass spectrometry, α-cyano-4-hydroxycinnamic acid (CHCA) was deposited as a matrix. Mass spectra were obtained with MALDI mass spectrometer (Thermo Scientific, MALDI-LTQ-Orbitrap). MS spectra were obtained with resolution at 30000, and with scan range of *m/z* 800-4,000. For MS/MS analysis, target peptide was fragmented with higher energy collisions (HCD) cell. MS/MS spectrum was obtained at mass range of *m/z* 200-2600 at resolution of 15000.

Sample preparation of Alt-AOMK-labeled CatB with and without click reaction using Alexa Fluor® 488 Azide, and its trypsin-digested peptide mixture (Figure 7 and Figure S22-S23).

Human liver CatB (10 μg) was treated with Alt-AOMK, using the same labeling and precipitation procedure as described above. The precipitate was dissolved in 10 μL of denaturing butter (7 M GuHCl, 1 M Tris-HCl (pH 8.5)), and was treated with Click-iT® protein reaction buffer kit (C10276, Invitrogen) according to the supplier's protocol. To the sample solution, 100 μL of 40 μM Alexa Fluor® 488 Azide (A10266, Invitrogen) and 40 μL of water were added and mixed, then 10 μL of $CuSO_4$ (component B) was added. Subsequently, 10 μL of additive-1 solution was added and mixed. After 3 min, 20 μL of additive-2 solution was added, and then the sample solution was mixed for 20 min with a rotator. Then, 600 μL of methanol, 150 μL of chloroform, and 400 μL of water were added sequentially to the sample solution. After centrifugation at 18000 G for 5 min, the supernatant was removed. The precipitate was washed with 450 μL of methanol twice and dried for 15 min. The precipitate was dissolved in 20 μL of denaturing buffer (7 M GuHCl, 1 M Tris-HCl (pH 8.5)) and then incubated for 1 hour at 37 °C. Subsequent reduction, alkylation, trypsin digestion and freeze-drying were performed according to the same procedure as described above.

Using the same protocol but without click reaction, trypsin-digested CatB labeled with Alt-AOMK was prepared for comparison.

For SDS-PAGE, 1 μL of sample solution from 20 μL of denaturing buffer with and without click-reaction was taken from the tube, and mixed with 50 μL of 1x Laemmli sample buffer. The mixture was boiled at 95 °C for 5 min. SDS-PAGE was performed at 150 V for around 40 min with 4-20% precast gel (MiniproteanTGX precast gels, BIORAD).

Fluorescence images of the gel were obtained with a laser-scanning fluorescence imager (Pharos FX, BIORAD) with excitation at 488 nm. CBB-stained gel was observed with a transmission-type imager (LAS-4000, Fuji Film).

Comparison between SERS detection and click-chemistry-based fluorescence detection of trypsin-digested peptide of Alt-AOMK-labeled CatB (Figure 7 and Figure S24-S25)

Trypsin-digested peptide mixture of Alt-AOMK-labeled CatB with and without click reaction using Alexa Fluor® 488 Azide were each fractionated on the Nano Frontier nLC. Columns, mobile phases and gradient for fractionation of peptides were the same as described for the experiment on trypsin-digested peptides of Alt-AOMK-labeled CatB.

For SERS detection using the sample without click-reaction, the eluate was fractionated into a glass-bottomed 384-well plate, preloaded with 25 μL per well of 0.3% (v/v) TFA solution, by a fraction collector at 20-sec intervals. The 25 μL solution was divided into two parts: 15 μL (for SERS) and 10 μL (for LC-MS). In the case of the 15 μL solution for SERS, 15 μL of silver nanoparticle solution was added to each well and the plate was left for for 1 day in the refrigerator at 4 °C. The plate was then placed on the Raman microscope stage. Experimental parameters and procedures were the same as in the protocol described for LC-SERS analysis of alkyne-tagged synthetic peptides. The SERS spectrum of each well was obtained by averaging SERS images (1 line, 1 x 400 pixels). Alkyne intensity was calculated as the difference between alkyne peak height at 1981 cm^{-1} and the peak bottom intensity.

In the case of fluorescence detection with click-reaction, the eluate was deposited on the MALDI target (ITOP plate, Thermo Scientific) via a fraction collector (Probot, Dionnex) at 20-sec intervals. Near the apex of the spotting capillary, 0.5 μL of water was added to each fraction to push the droplet onto the substrate. After the droplets had dried, a fluorescence imager (Pharos FX, BIORAD) set for excitation at 488 nm (530 nm detection) was used to obtain fluorescence images. An intensity plot was calculated from the maximum intensity of each spot to generate the fluorescence chromatogram. For comparison with the SERS chromatogram, retention time was adjusted by approximately 2.5 min according to the peak positions in the UV chromatogram.

Target spots indicated by the fluorescence imaging were recovered and subjected to LC-MS.

Cathepsin Inhibition Assay

The cathepsin-inhibitory activities were measured by using fluorometric assay kits (BioVision, K140-100 for CatB, K142-100 for cathepsin L (CatL), and K144-100 for cathepsin S (CatS)) and purified cathepsins (CALBIOCHEM, human liver CatB, human liver CatL, and recombinant CatS). In each well of a 96-well plate, fluorogenic substrate for each cathepsin (100 μM) and test compound (0.1-30 μM) were mixed in cathepsin reaction buffer (50 mM sodium acetate (pH 5.6), 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT)), and then cathepsin was added (CatB 5 ng/well, CatL 5 ng/mL, CatS 1 ng/well, and final volume 100 μL/well). The plate was incubated at 37 ^oC for 30 min, and the cathepsin activity was determined based on the increase of fluorescence (Ex 400 nm / Em 505 nm) measured with a 96-well plate reader (Spectra Max M2e, Molecular Devices). The dose-response curves and IC_{50} values of compounds were calculated by Origin 9.0 software.

Sample preparation of Alt-AOMK-labeled CatB and its trypsin-digested peptide mixture (Figure S21).

Human liver CatB (10 μg, CALBIOCHEM) was dissolved in 100 μL of buffer (50 mM sodium acetate (pH 5.6), 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT)), and the solution was kept for 15 min at room temperature. It was then mixed with 1.0 μL of 20 mM Alt-AOMK dissolved in DMSO, and the mixture was incubated for 3 hours at 37 °C. After incubation, the proteins in the solution were purified by trichloroacetic acid (TCA) precipitation, with an incubation time of 3 hours on ice. Centrifugation was performed at 20000 G for 20 min to obtain the precipitate. After removal of the supernatant, 1 mL of acetone was added, and centrifugation was performed at 20000 G for 15 min. After removal of acetone, 1 mL of acetone was added again. Centrifugation and acetone treatment was performed three times. The remaining acetone was removed in vacuo for 30 sec. The precipitate was dissolved in 20 μL of denaturing buffer (7 M GuHCl, 500 mM Tris-HCl (pH 8.5)), and the solution was incubated for 1 hour at 37 °C. After reduction and alkylation with DTT and iodoacetamide trypsin at a concentration of 100 ng/μL was added

and incubated for several hours at 37 °C. Approximately 0.01% (w/v) *n*-decyl-β-D-glucopyranoside (DG) (240 μL) was added. From one-tenth to one-thousandth of the total solution was loaded in the HPLC, which corresponds to the injection volume of CatB from 1.0 μg to 0.01 μg. 0.01% DG solution was used for control, which corresponds to the injection volume of CatB at 0 μg.

LC-SERS analysis of trypsin-digested peptide of Alt-AOMK-labeled CatB (Figure S21).

Trypsin-digested peptide mixture of Alt-AOMK-labeled CatB was fractionated on an UltiMate 3000 RSLC nano system (Thermo scientific) equipped with a MU701 UV detector. Peptides were separated on an Acclaim PepMap RSLC (C18, 2 μm, 0.075 x 150 mm, Thermo scientific) with an Acclaim PepMap μ-precolumn (C18, 5 μm, 0.3 x 5 mm, Thermo scientific) at flow rate of 250 nL/min. The UV chromatogram was obtained with a UV detector (MU701, GL Science) set at 215 nm. For the analytical column, mobile phase A (distilled water containing 0.1% (v/v) TFA) and mobile phase B (0.1% (v/v) TFA and 90% (v/v) MeCN) were utilized to prepare a gradient as follows. 0-10 min: 4% B, 10-35 min: 4-39% B, 35-45 min: 39-78% B, 45-45.1 min: 78-99% B, 45.1-49 min: 99% B, 49-50 min: 99-4% B, 50-60 min: 4% B. The eluate was fractionated into a glass-bottomed 384-well plate, preloaded with 25 μL of 0.3% (v/v) TFA solution in each well, via a fraction collector at 20 sec intervals. 30 μL of silver nanoparticle solution was added to each well, and the plate was kept for 1 day in the refrigerator at 4 °C. The 384-well plate was set on the Raman microscope stage. Experimental parameters and procedures were the same as in the protocol described for LC-SERS analysis of alkyne-tagged synthetic peptide, except for the laser power, which was set at 460 mW, and exposure time, which was set at 5 sec/line (1 x 400 pix). The SERS spectrum of each well was obtained by averaging SERS images (1 line, 1 x 400 pixels). Alkyne intensity was calculated as the difference of alkyne peak height at 1981 cm^{-1} and peak bottom intensity.

Sample preparation of cell lysate with CatB and its trypsin-digested peptide mixture (Figure S27).

Cell lysate was prepared from HL-60 cells. HL-60 cells (4.2×10^7 cells) were collected, washed with PBS, then lysed by 1.4 mL of cell lysis buffer (cathepsin B activity fluorometric assay kit, K140-100, BioVision). After centrifugation at 15000 G for 10 min, supernatant was obtained as cell lysate, in which protein concentration was determined by Quick Start Bradford Assay kit (BIORAD) as 0.93 mg/mL. 22.5 μg, 24.5 μg and 49.5 μg of cell lysate solution were mixed with 2.5 μg, 0.5 μg and 0.5 μg of CatB (CALBIOCHEM) respectively. Each sample includes CatB at a tenth part (sample A), fiftieth part (sample B) and a hundredth part (sample C) of total proteins. Each sample is dissolved in 100 μL of buffer (50 mM sodium acetate (pH 5.6), 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT)), and the solution was kept for 15 min at room temperature. It was then mixed with 1.0 μL of 20 mM Alt-AOMK dissolved in DMSO, and

the mixture was incubated for 3 hours at 37 °C. After incubation, the proteins in the solution were purified by trichloroacetic acid (TCA) precipitation, with an incubation time of 3 hours on ice. Centrifugation was performed at 20000 G for 20 min to obtain the precipitate. After removal of the supernatant, 1 mL of acetone was added, and centrifugation was performed at 20000 G for 15 min. After removal of acetone, 1 mL of acetone was added again. Centrifugation and acetone treatment was performed three times. The remaining acetone was removed in vacuo for 30 sec. The precipitate was dissolved in 20 μL of denaturing buffer (7 M GuHCl, 500 mM Tris-HCl (pH 8.5)), and the solution was incubated for 1 hour at 37 °C. After reduction and alkylation with DTT and iodoacetamide, trypsin at a concentration of 100 ng/μL was added and incubated for several hours at 37 °C. Approximately 0.01% (w/v) *n*-decyl-β-D-glucopyranoside (DG) (240 μL) was added. For sample A and B, one-tenth of the total solution was used for LC-fractionation. For sample C, one-twentieth of the total solution was used for LC-fractionation.

LC-SERS-MS analysis of trypsin-digested peptide of cell lysate with CatB (Figure S27, Table S2).

Trypsin-digested peptide mixture of cell lysate containing CatB (Sample A, B, and C described above) was fractionated on an UltiMate 3000 RSLC nano system (Thermo scientific) equipped with a MU701 UV detector. Peptides were separated on an Acclaim PepMap RSLC (C18, 2 μm, 0.075 x 150 mm, Thermo scientific) with an Acclaim PepMap μ-precolumn (C18, 5 μm, 0.3 x 5 mm, Thermo scientific) at flow rate of 250 nL/min. The UV chromatogram was obtained with a UV detector (MU701, GL Science) set at 215 nm. For the analytical column, mobile phase A (distilled water containing 0.1% (v/v) TFA) and mobile phase B (0.1% (v/v) TFA and 90% (v/v) MeCN) were utilized to prepare a gradient as follows. 0-10 min: 4% B, 10-35 min: 4-39% B, 35-45 min: 39-78% B, 45-45.1 min: 78-99% B, 45.1-49 min: 99% B, 49-50 min: 99-4% B, 50-60 min: 4% B. The eluate was fractionated into a glass-bottomed 384-well plate, preloaded with 25 μL of 0.3% (v/v) TFA solution in each well, via a fraction collector at 20 sec intervals. 30 μL of silver nanoparticle solution was added to each well, and the plate was kept for 1 day in the refrigerator at 4 °C. The 384-well plate was set on the Raman microscope stage. Experimental parameters and procedures were the same as in the protocol described for LC-SERS analysis of alkyne-tagged synthetic peptide, except for the laser power, which was set at 460 mW, and exposure time, which was set at 5 sec/line (1 x 400 pix). The SERS spectrum of each well was obtained by averaging SERS images (1 line, 1 x 400 pixels). Alkyne intensity was calculated as the difference of alkyne peak height at 1981 cm^{-1} and peak bottom intensity. For MALDI-TOF-MS, supernatant of the target fraction was removed, and 1 μl of 0.01% DG and 1 μL of MeCN was added in the well. The precipitated sample on the substrate was collected in the solution by scratching the surface of the substrate with a pipette tip. All of the sample solution was deposited on the MALDI plate, and analyzed by using an autoflex speed TOF/TOF mass spectrometer (Bruker Daltonics) in positive and reflector mode. *α*-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics) was used as the matrix.

Direct MALDI-TOF MS analysis of tryptic digest of the Alt-AOMK-treated cell lysate containing CatB (Figure S28).

Trypsin-digested peptide mixture of cell lysate mixed with CatB (Sample A, B and C described in Figure S27) was desalted and concentrated with a ZipTip C_{18} (Merck Millipore). Loading amount for each sample was 2.5 μg, which is the same as the amount loaded in the HPLC for ATRaS measurement. After the elution step, all of the eluate was deposited on a MALDI plate and analyzed by using an autoflex speed TOF/TOF in positive and reflector mode. α-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics) was used as the matrix.

Direct LC-ESI MS analysis of tryptic digest of the Alt-AOMK-treated cell lysate containing CatB (Figure S29).

LC-MS/MS analyses were performed using a LTQ Orbitrap XL mass spectrometer (Thermo Fisher Scientific) equipped with a nanospray stage (Nikkyo Technos) and an UltiMate 3000 nano HPLC system (Thermo Fisher Scientific). Tryptic digest of the Alt-AOMK-treated HL60 cell lysate mixed with CatB at (A) a tenth part, (B) fiftieth part and (C) a hundredth part of total proteins was injected into an HPLC system and separated on an Acclaim PepMap100 nanoViper column (0.075 x 150 mm, Thermo Fisher Scientific) after trapping and desalting with a μ-precolumn C18 PepMap100 (0.3 x 5 mm, Thermo Fisher Scientific). 2 μL of each original sample solution was used, corresponding to the loading amount of the total protein volume at approximately 0.2 μg for A and B, and at approximately 0.4 μg for C. Fused-silica tips (PicoTip Emitter silica tip, FS360-20-10-N, New Objective) was used as an electrospray emitter. Elution of peptides was carried out using a gradient as follows. 0-10 min: 0% B, 10-44 min: 4-60% B, 44-45 min: 60-90% B, 45-50 min: 90% B (A: distilled water containing 0.1% (v/v) FA and 4% (v/v) MeCN, B: MeCN containing 0.1% (v/v) FA) at a flow rate of 250 nL/min.

LC-MS/MS data was obtained by using data-dependent acquisition (DDA) and positive ion mode. Full MS scan (m/z 350-1,600) was acquired in the Orbitrap at a resolution setting of 60,000 and target automatic gain control (AGC) value of 5×10^5, and CID-MS/MS was performed on the 10 most intense ions in the ion trap.

MS/MS data were submitted for database searching by the MASCOT (www.matrixscience.com) search engine installed in-house via Proteome Discoverer software (Thermo Fisher Scientific). The MS/MS ion search parameters were as follows: database; NCBInr Homo sapiens; +/- 0.8 Da fragment mass tolerance; +/- 5 ppm peptide mass tolerance; trypsin enzyme specificity (up to three missed cleavages); variable modifications of methionine oxidation (15.99491 Da), carbamidomethylation on cysteine (57.02146 Da), and Alt-AOMK on cysteine (376.142307 Da). According to the MASCOT search result, $[M+3H]^{3+}$ of Alt-AOMK-labeled peptide ^{22}DQGSCGSCWAFGAVEAISDR^{41} was identified for sample A, while was not identified for sample B and C.

Sample preparation of Alt-AOMK-labeled CatB, CatL, and CatS, and its tryptic digest (Figure S30-S31).

Human liver CatB (2.5 μg, CALBIOCHEM), human liver CatL (2.5 μg, CALBIOCHEM) or human recombinant CatS (2.5 μg, CALBIOCHEM) was dissolved in 100 μL of buffer (50 mM sodium acetate (pH 5.6), 5 mM $MgCl_2$, 2 mM dithiothreitol (DTT)), and the solution was kept for 15 min at room temperature. They were then mixed with 1.0 μL of 20 mM Alt-AOMK dissolved in DMSO, and the mixture was incubated for 3 hours at 37 °C. After incubation, the proteins in the solution were purified by trichloroacetic acid (TCA) precipitation, with an incubation time of 3 hours on ice. Centrifugation was performed at 20000 G for 20 min to obtain the precipitate. After removal of the supernatant, 1 mL of acetone was added, and centrifugation was performed at 20000 G for 15 min. After removal of acetone, 1 mL of acetone was added again. Centrifugation and acetone treatment was performed three times. The remaining acetone was removed in vacuo for 30 sec. The precipitate was dissolved in 20 μL of denaturing buffer (7 M GuHCl, 500 mM Tris-HCl (pH 8.5)), and the solution was incubated for 1 hour at 37 °C. After reduction and alkylation with DTT and iodoacetamide trypsin at a concentration of 100 ng/μL was added and incubated for several hours at 37 °C. Approximately 0.01% (w/v) *n*-decyl-β-D-glucopyranoside (DG) (240 μL) was added. For each sample, 24 % of the total solution was loaded in the HPLC, which corresponds to the injection volume of CatB, CatL, and CatS at 0.6 μg. For CatL, 48 % of the total solution was also loaded in the HPLC, which corresponds to the injection volume of 1.2 μg. The sample solution of tryptic digest of Alt-AOMK-treated CatL was analyzed by LC-MS with loading amount at 9.6 ng, and peaks corresponding to the $[M+3H]^{3+}$ of Alt-MK-labeled peptide ^{131}NQGQCGSCWAFSATGALEGQMFR^{153} were not observed. Instead, MS spectrum of $[M+3H]^{3+}$ of peptide ^{131}NQGQCGSCWAFSATGALEGQMFR^{153}, whose two cysteine residues were both modified with carbamidomethyl, was observed indicating that labeling efficiency of CatL with Alt-AOMK was quite low.

LC-SERS-MS analysis of trypsin-digested peptide of Alt-AOMK-treated CatB, CatL, and CatS (Figure S30-S31, Table S3).

Trypsin-digested peptide mixture of CatB, CatL, and CatS was fractionated on an UltiMate 3000 RSLC nano system (Thermo scientific) equipped with a MU701 UV detector. Peptides were separated on an Acclaim PepMap RSLC (C18, 2 μm, 0.075 x 150 mm, Thermo scientific) with an Acclaim PepMap μ-precolumn (C18, 5 μm, 0.3 x 5 mm, Thermo scientific) at flow rate of 250 nL/min. The UV chromatogram was obtained with a UV detector (MU701, GL Science) set at 215 nm. For the analytical column, mobile phase A (distilled water containing 0.1% (v/v) TFA) and mobile phase B (0.1% (v/v) TFA and 90% (v/v) MeCN) were utilized to prepare a gradient as follows. 0-10 min: 4% B, 10-35 min: 4-39% B, 35-45 min: 39-78% B, 45-45.1 min: 78-99% B, 45.1-49 min: 99% B, 49-50 min: 99-4% B,

50-60 min: 4% B. The eluate was fractionated into a glass-bottomed 384-well plate, preloaded with 25 μL of 0.3% (v/v) TFA solution in each well, via a fraction collector at 20 sec intervals. 30 μL of silver nanoparticle solution was added to each well, and the plate was kept for 1 day in the refrigerator at 4 °C. The 384-well plate was set on the Raman microscope stage. Experimental parameters and procedures were the same as in the protocol described for LC-SERS analysis of alkyne-tagged synthetic peptide, except for the laser power, which was set at 460 mW, and exposure time, which was set at 5 sec/line (1 x 400 pix). The SERS spectrum of each well was obtained by averaging SERS images (1 line, 1 x 400 pixels). Alkyne intensity was calculated as the difference of alkyne peak height at 1981 cm^{-1} and peak bottom intensity. For MALDI-MS, supernatant of the target fraction was removed, and 1 μl of 0.01% DG and 1 μL of MeCN were added to the well. The precipitated sample on the substrate was collected in the solution by scratching the surface of the substrate with a pipette tip. All of the sample solution was deposited on the MALDI plate, and analyzed by using an autoflex speed TOF/TOF mass spectrometer (Bruker Daltonics) in positive and reflector mode. *α*-cyano-4-hydroxycinnamic acid (CHCA, Bruker Daltonics) was used as the matrix.

Supplementary Table

amino-acid	y-ion	observed mass (m/z)	mass error (ppm)	modification
D	20	---	---	
Q	19	2377.0041	1.220	
G	18	2248.9526	4.402	
S	17	---	---	
C + CAM	16	2104.8940	2.280	+ Carbamidomethylation
G	15	1944.8630	2.314	
S	14	1887.8456	4.555	
C + Alt-MK	13- H_2O	1782.7985	2.244	+ Alt-MK
W	12	1321.6562	2.043	
A	11	1135.5769	2.378	
F	10	1064.5390	1.785	
G	9	917.4707	2.180	
A	8	860.4496	2.789	
V	7	789.4121	2.534	
E	6	690.3437	2.879	
A	5	561.3005	2.494	
I	4	490.2634	2.856	
S	3	377.1792	3.447	
D	2	290.1470	3.791	
R	1	---	---	

Table S1. Observed product ions of Alt-MK-modified peptide [DQGSC^{26}(CAM)GSC^{29}(Alt-AOMK)WAFGAVEAISDR]. CAM is the abbreviation of carbamidomethylation.

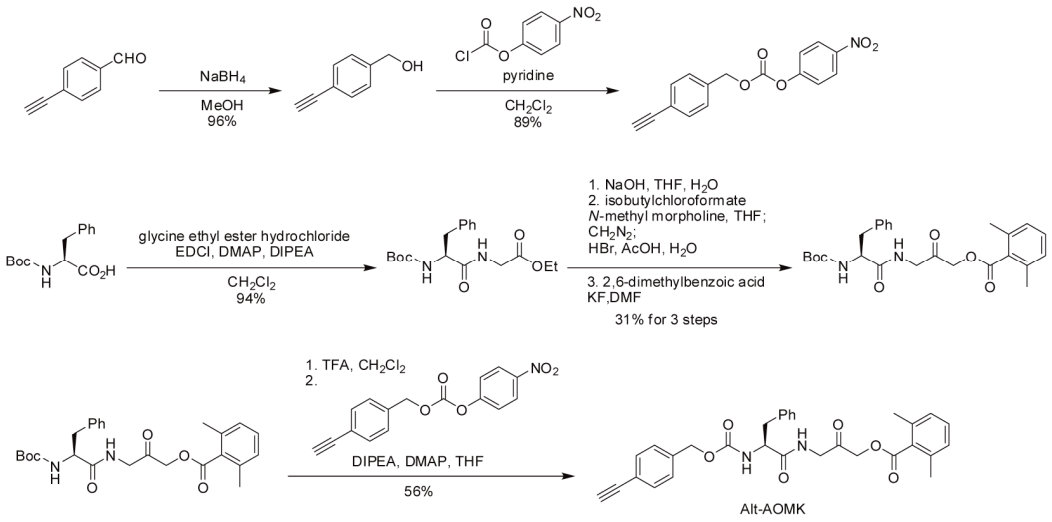
fraction #	sequence positions	sequence	modification on Cys	calculated[M+H]$^+$ (*m/z*)	observed [M+H]$^+$ (*m/z*)	*m/z* error (ppm)
Fraction I	22-41	DQGSCGSCWAFGAVEAISDR	CAM, Alt-MK	2492.028	2492.031	1.1
Fraction II	22-41	DQGSCGSCWAFGAVEAISDR	CAM, Alt-MK	2492.028	2492.024	-1.6
Fraction III	22-41	DQGSCGSCWAFGAVEAISDR	CAM, Alt-MK	2492.028	2492.002	-10.6

Table S2. List of peptides detected by MALDI-TOF MS in target fractions in Figure S27. Underbars in the sequence indicate the positions of modified residues. CAM is the abbreviation for carbamidomethylation.

fraction #	sequence positions	sequence	modification on Cys	calculated[M+H]$^+$ (*m/z*)	observed [M+H]$^+$ (*m/z*)	*m/z* error (ppm)
CatB						
Fraction IV	19-41	EIRDQGSCGSCWAFGAVEAISDR	CAM, Alt-MK	2890.26	2890.30	14.7
Fraction V	22-41	DQGSCGSCWAFGAVEAISDR	CAM, Alt-MK	2492.03	2492.01	-8.3
CatS						
Fraction VI	125-153	GCVTEVKYQGSCGACWAFSAVGALEAQLK	CAM (x2), Alt-MK	3466.59	3466.44	-42.1
Fraction VII	132-153	YQGSCGACWAFSAVGALEAQLK	CAM, Alt-MK	2693.22	2693.24	7.1

Table S3 List of Alt-MK modified peptides in target fractions (Figure S31) detected by MALDI-TOF MS analysis. Underbars in the sequence indicate the positions of modified residues. CAM is the abbreviation for carbamidomethylation.

Supplementary Synthetic Scheme



Scheme S1. Scheme for the synthesis of Alt-AOMK.

Supplementary Synthetic Procedures and Compounds Data

Syntheses of compounds (general)

Nuclear magnetic resonance (NMR) spectra were recorded on JEOL 400 or 300 instruments, and ^{1}H-NMR data are reported with chemical shifts quoted in parts per million (δ p.p.m.) downfield relative to tetramethylsilane (TMS). Coupling constants (*J*) are reported in Hz. Multiplicities are reported using the following abbreviations: s, singlet; d, doublet; t, triplet; q; quartet; m, multiplet; br, broad. Melting points were determined on a Yanaco MP-J8 melting point apparatus. Mass spectra were recorded on Bruker microTOF-QII-RSL. Optical rotations were measured on a JASCO DIP-370 digital polarimeter at room temperature using the sodium D line. Flash column chromatography with standard silica gel (Silica gel 60N, spherical, neutral, 100 ~ 210 μm, Kanto Chemical Co. Ltd.). Gel filtration chromatography was performed with JAI LC-918. Anis stain solution (2.5% (v/v) anisaldehyde, 3.5% (v/v) of H_2SO_4, and 1% (v/v) of acetic acid in ethanol) was used for monitoring and visualizing TLC bands. Distilled solvents (MeOH, THF, CH_2Cl_2, and DMF) were purchased from Kanto Chemical Co. Ltd. and used as received, and other reagents were used without any purification. Reactions were performed at ambient temperature unless otherwise stated.

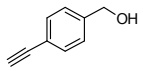
Peptide synthesis

The peptides were prepared by solid-phase synthesis (433A Peptide Synthesizer, Applied Biosystems) using Fmoc-protected amino acids. Synthesized peptides were purified by HPLC (L-2000, Hitachi) to >90% purity. HPLC

Conditions: Inertsil ODS-3 (250 x 4.6 mm I.D.) as column, 15-45% CH_3CN (contg. 0.1% TFA) (30 min) as mobile phase, 1.0 mL/min as flow rate and UV at 215 nm as light source.

Experimental procedures and compounds data

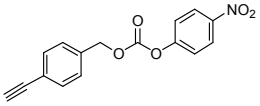
4-Ethynylbenzyl alcohol



To a solution of 4-ethynylbenzaldehyde (300 mg, 2.31 mmol) in MeOH was added $NaBH_4$ (61 mg, 1.61 mmol) at 0 °C and the mixture was stirred for 5 min. The reaction mixture was quenched with 2 N aqueous HCl, then H_2O was added and the whole was extracted with $CHCl_3$ two times. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude mixture was purified by flash silica gel column chromatography (AcOEt / hexane = 3 / 2) to give 4-ethynylbenzyl alcohol (294 mg, 96%) as a colorless solid.

Colorless solid; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.49 (2H, d, *J* = 8.1 Hz), 7.32 (2H, d, *J* = 8.1 Hz), 4.70 (2H, d, *J* = 5.6 Hz), 3.07 (1H, s), 1.71 (1H, t, *J* = 5.6 Hz); ^{13}C-NMR (75 MHz, $CDCl_3$) δ: 141.5, 132.1, 126.6, 121.1, 83.4, 77.2, 64.5; MS (ESI) *m/z*: 155 [$(M+Na)^+$]; HRMS (ESI) Calcd. for C_9H_8ONa: 155.0467. Found: 155.0479.

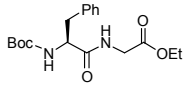
4-Nitrophenyl-4-ethynylbenzyl carbonate



To a solution of 4-ethynylbenzyl alcohol (168 mg, 1.27 mmol) in CH_2Cl_2 (5.8 mL) were added pyridine (0.28 mL, 3.48 mmol) and 4-nitrophenyl chloroformate (233 mg, 1.16 mmol) at 0 °C. The reaction mixture was stirred at room temperature for 4 hours, then saturated aqueous $NaHCO_3$ was added and the mixture was extracted with $CHCl_3$ two times. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude mixture was purified by flash silica gel column chromatography (AcOEt / hexane = 1 / 4) to give 4-nitrophenyl-4-ethynylbenzyl carbonate (306 mg, 89%) as a colorless solid.

Colorless needles (AcOEt / hexane = 1 / 4): mp 141 – 143 °C; ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 8.28 (2H, d, *J* = 9.2 Hz), 7.54 (2H, d, *J* = 8.0 Hz), 7.40 (2H, d, *J* = 8.0 Hz), 7.38 (2H, d, *J* = 9.2 Hz), 5.29 (2H, s), 3.13 (1H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 155.4, 152.4, 145.4, 134.7, 132.5, 128.4, 125.3, 122.9, 121.7, 82.9, 78.2, 70.3; MS (ESI) *m/z*: 320 [$(M+Na)^+$]; HRMS (ESI) Calcd. for $C_{16}H_{11}NO_5Na$: 320.0529. Found: 320.0530.

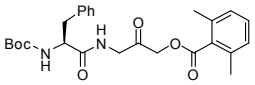
Ethyl 2-(2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanamido)acetate



To a mixture of 2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanoic acid (280 mg, 1.06 mmol) and glycine ethyl ester hydrochloride (192 mg, 1.37 mmol) in CH_2Cl_2 (5.3 mL) were added 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (263 mg, 1.37 mmol), 4,4-dimethylaminopyridine (13 mg, 0.11 mmol), and diisopropylethylamine (0.54 mL, 3.18 mmol). The mixture was stirred for 14 hours, and then H_2O and AcOEt were added. The layers were separated and the aqueous phase was extracted with AcOEt three times. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude mixture was purified by flash silica gel column chromatography (AcOEt / hexane = 4 / 1) to give ethyl 2-(2-((*tert*-butoxycarbonyl)amino)-3-phenyl propanamido)acetate (350 mg, 94%) as a colorless solid.

Colorless solid; $[\alpha]_D^{23}$ –4.38 (*c* 0.966, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.32-7.20 (5H, m), 6.53 (1H, brs), 5.07 (1H, brs), 4.43 (1H, brs), 4.19 (2H, q, *J* = 7.1 Hz), 4.02 (1H, dd, *J* = 18.4, 5.5 Hz), 3.94 (1H, dd, *J* = 18.4, 5.0 Hz), 3.13 (1H, dd, *J* = 13.8, 5.5 Hz), 3.04 (1H, dd, *J* = 13.8, 6.0 Hz), 1.39 (9H, s), 1.27 (3H, t, *J* = 7.1 Hz); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 171.5, 169.4, 155.4, 136.6, 129.3, 128.6, 126.9, 80.2, 61.5, 55.6, 41.3, 38.3, 28.2, 14.1; MS (ESI) *m/z*: 373 [$(M+Na)^+$]; HRMS (ESI) Calcd. for $C_{18}H_{26}N_2O_5Na$: 373.1734. Found: 373.1743.

3-(2-((*tert*-Butoxycarbonyl)amino)-3-phenylpropanamide)-2-oxopropyl 2,6-dimethylbenzoate



To a solution of ethyl 2-(2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanamido)acetate (2.0 g, 5.7 mmol) in THF (29 mL) and MeOH (29 mL) was added 10% aqueous NaOH (19 mL). The mixture was stirred for 10 min at 0 °C, then quenched with 7.5% aqueous HCl, and the whole was extracted with CH_2Cl_2 six times. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure.

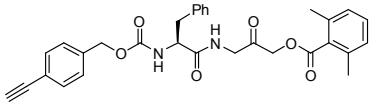
To a solution of the residue in THF (27 mL) at –10 °C were sequentially added *N*-methylmorpholine (970 μL, 8.8 mmol) and isobutyl chloroformate (1.05 mL, 8.1 mmol). The solution was stirred for 30 min at –10 °C, followed by addition of excess ethereal diazomethane. The solution was warmed to room temperature over 3 hours with stirring. A solution of 33% HBr in AcOH (10.5 mL) and H_2O (10.5 mL) was added dropwise and resulting solution was stirred for an additional 10 min at 0 °C. The reaction was stopped by addition of aqueous saturated $NaHCO_3$ and AcOEt. The layers were separated and the aqueous phase was extracted with AcOEt two times. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude mixture was purified by flash silica gel column chromatography (AcOEt / hexane = 2 / 1) to give bromomethyl ketone (1.55 g, 57%) as a colorless amorphous solid.

To a solution of bromomethyl ketone (1.5 g, 3.76 mmol) in DMF (9.4 mL) were added potassium fluoride (874 mg, 15.0 mmol) and 2,6-dimethylbenzoic acid (733 mg, 4.89 mmol). The mixture was stirred for 1 day at room

temperature, and the DMF solvent was removed under reduced pressure. To the crude mixture were added CH_2Cl_2 and H_2O. The aqueous phase was extracted with CH_2Cl_2 two times. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude mixture was purified by flash silica gel column chromatography (AcOEt / hexane = 1 / 1) to give 3-(2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanamide)-2-oxopropyl 2,6-dimethylbenzoate (952 mg, 54%) as a colorless amorphous solid.

Colorless amorphous solid; $[\alpha]_D^{26}$ –3.03 (*c* 0.760, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.30-7.19 (6H, m), 7.04 (2H, d, *J* = 7.8 Hz), 6.89 (1H, brs), 5.15 (1H, brs), 4.88 (2H, s), 4.47 (1H, brs), 4.22 (1H, dd, *J* = 19.3, 5.1 Hz), 4.15 (1H, dd, *J* = 19.3, 5.1 Hz), 3.12 (1H, dd, *J* = 13.8, 6.4 Hz), 3.03 (1H, dd, *J* = 13.8, 6.5 Hz), 2.37 (6H, s), 1.39 (9H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 198.7, 171.9, 168.9, 155.4, 136.4, 135.5, 132.2, 129.8, 129.2, 128.6, 127.7, 126.9, 80.3, 66.6, 55.6, 46.6, 38.2, 28.2, 19.8; MS (ESI) *m/z*: 491 [$(M+Na)^+$]; HRMS (ESI) Calcd. for $C_{26}H_{32}N_2O_6Na$: 491.2153. Found: 491.2165.

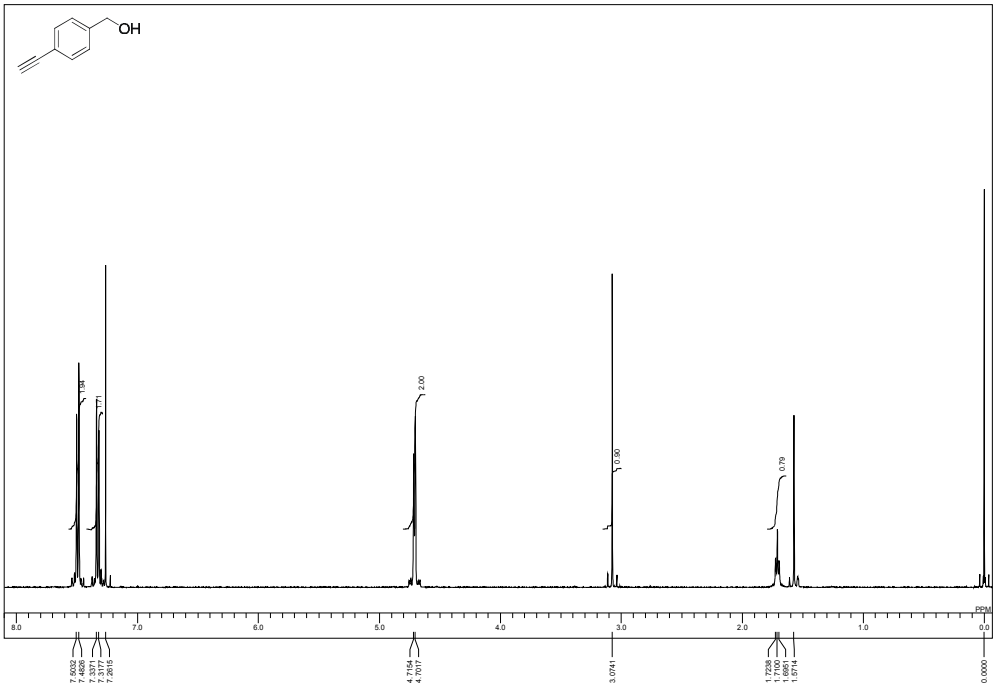
Alt-AOMK

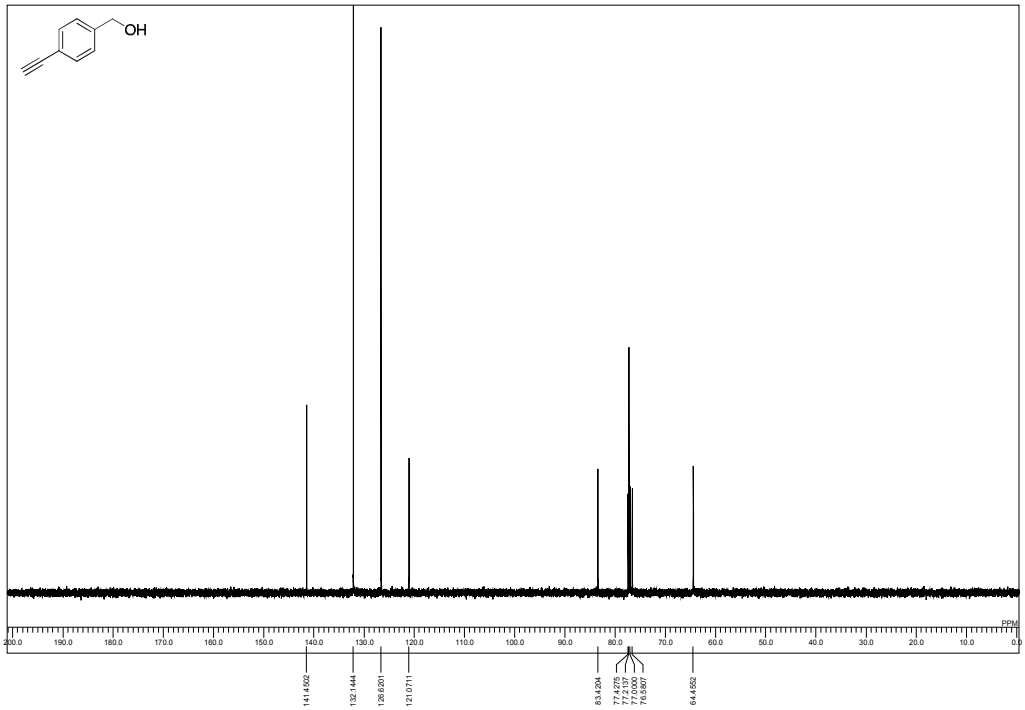


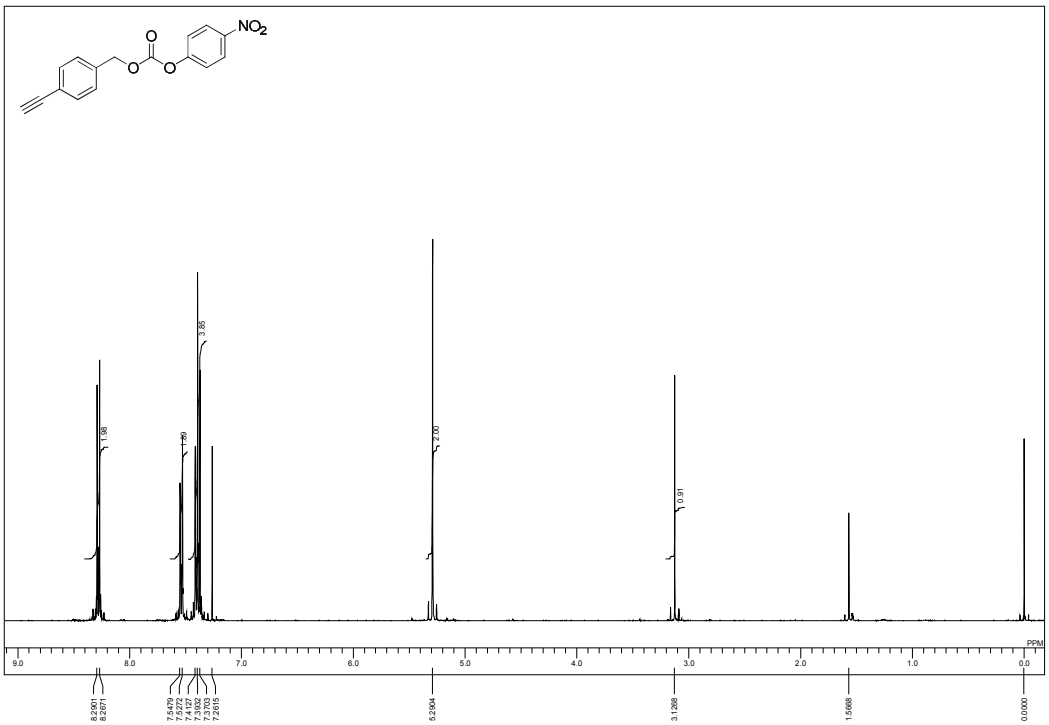
To a solution of 3-(2-((*tert*-butoxycarbonyl)amino)-3-phenylpropanamide)-2-oxopropyl 2,6-dimethylbenzoate (83 mg, 177 μmol) in CH_2Cl_2 (0.75 mL) was added TFA (0.25 mL). The mixture was stirred for 30 min, and the solvent was removed under reduced pressure. To a solution of the residue in THF (0.74 mL) were added 4-nitrophenyl 4-ethynylbenzyl carbonate (35 mg, 118 μmol), DIPEA (160 μL, 1.6 mmol), and 4-dimethylaminopyridine (14 mg, 0.12 mmol). The mixture was stirred for 2 hours at room temperature, and then H_2O and AcOEt were added. The layers were separated and the aqueous phase was extracted with AcOEt two times. The combined organic extracts were dried over $MgSO_4$, filtered, and concentrated under reduced pressure. The crude mixture was purified by flash silica gel column chromatography (AcOEt) followed by gel filtration chromatography to give Alt-AOMK (34.7 mg, 56%) as a colorless amorphous solid.

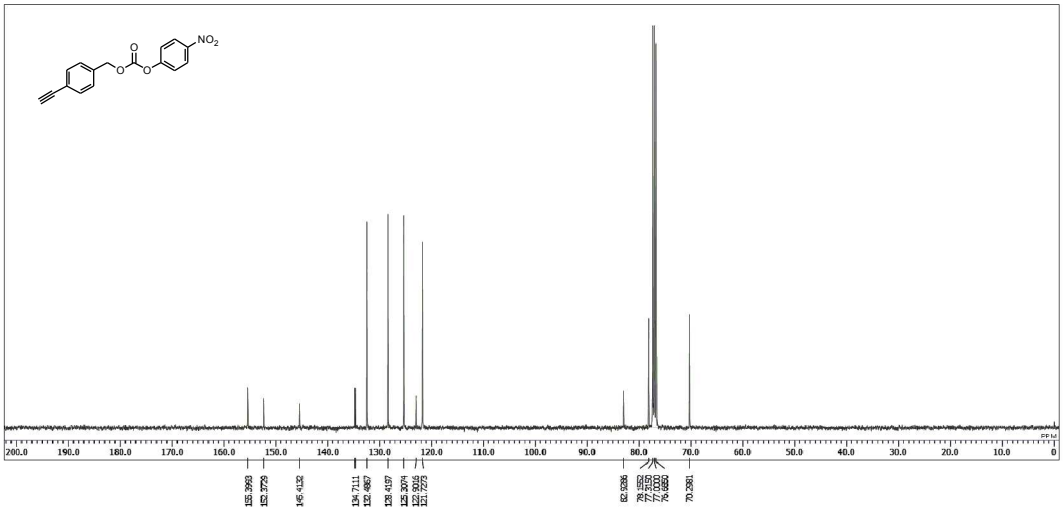
Colorless amorphous solid; $[\alpha]_D^{23}$ + 0.66 (*c* 0.915, $CHCl_3$); ^{1}H-NMR (400 MHz, $CDCl_3$) δ: 7.43 (2H, d, *J* = 8.3 Hz), 7.28-7.16 (8H, m), 7.04 (2H, d, *J* = 7.8 Hz), 6.75 (1H, brs), 5.50 (1H, brd, *J* = 7.2 Hz), 5.06 (1H, d, *J* = 12.7 Hz), 5.00 (1H, d, *J* = 12.7 Hz), 4.86 (2H, s), 4.53 (1H, m), 4.21 (1H, dd, *J* = 19.3, 5.3 Hz), 4.14 (1H, dd, *J* = 19.3, 4.8 Hz), 3.11 (1H, dd, *J* = 13.7, 6.8 Hz), 3.09 (1H, s), 3.04 (1H, m), 2.36 (6H, s); ^{13}C-NMR (100 MHz, $CDCl_3$) δ: 198.6, 171.3, 169.0, 155.8, 136.8, 136.1, 135.6, 132.2, 132.1, 129.9, 129.2, 128.7, 127.7, 127.7, 127.1, 121.9, 83.2, 77.6, 66.6, 66.5, 56.0, 46.5, 38.4, 19.9; MS (ESI) *m/z*: 549 [$(M+Na)^+$]; HRMS (ESI) Calcd. for $C_{31}H_{30}N_2O_6Na$: 549.1996. Found: 549.2012.

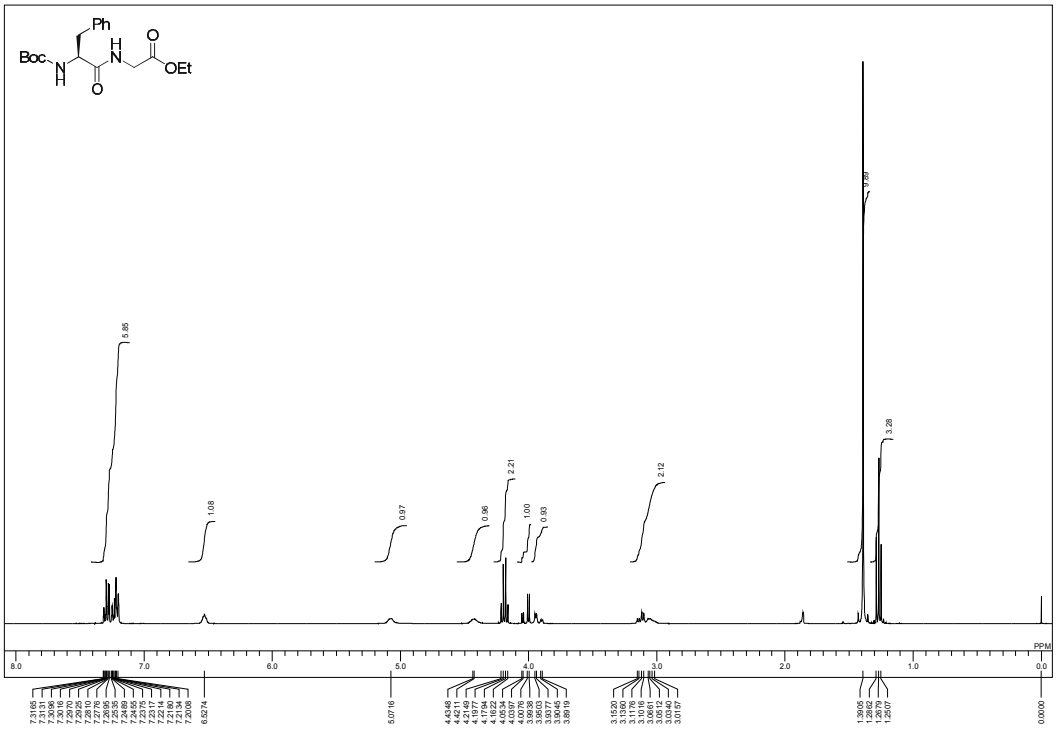
^{1}H and ^{13}C NMR Spectra of Compounds







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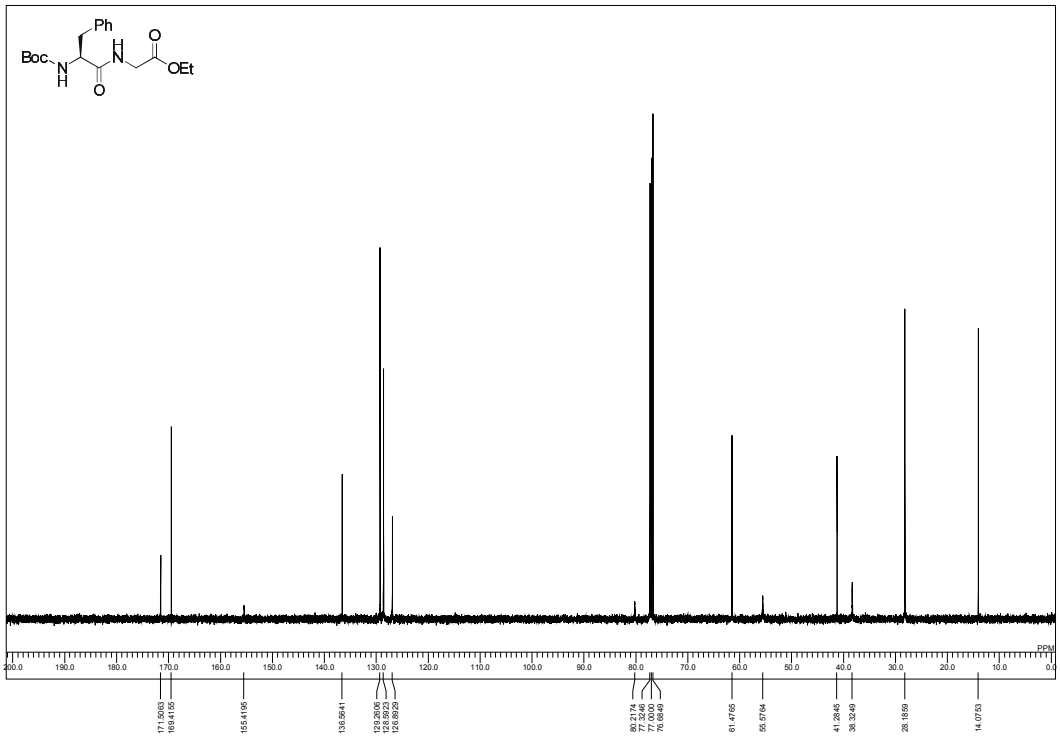
PPM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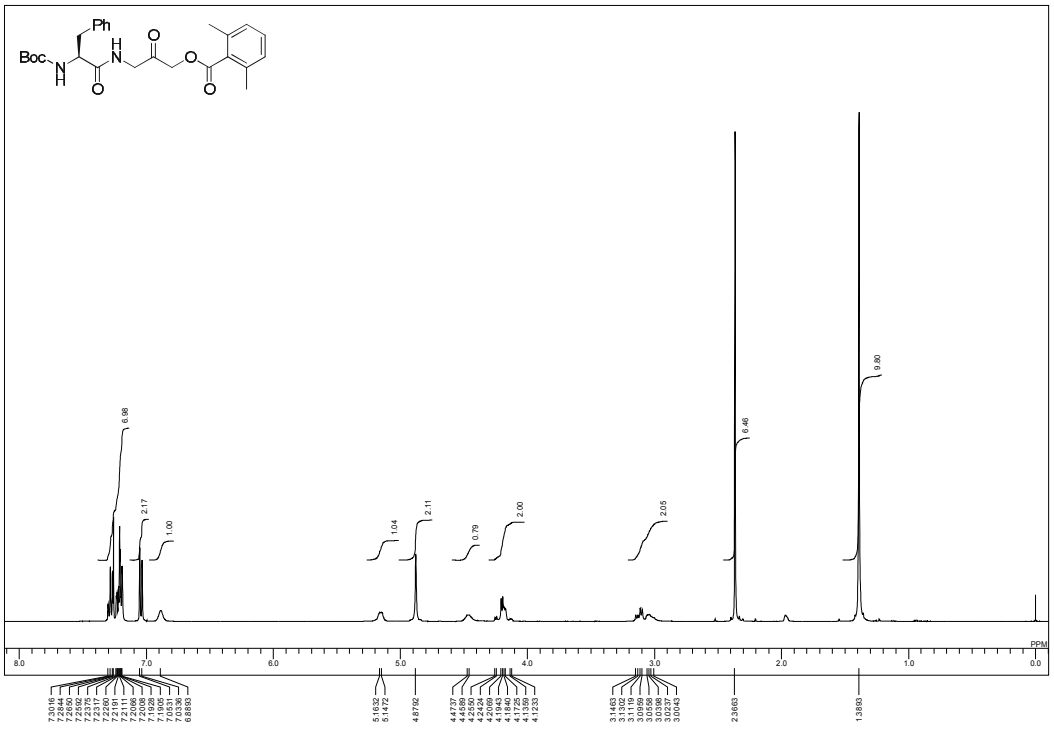
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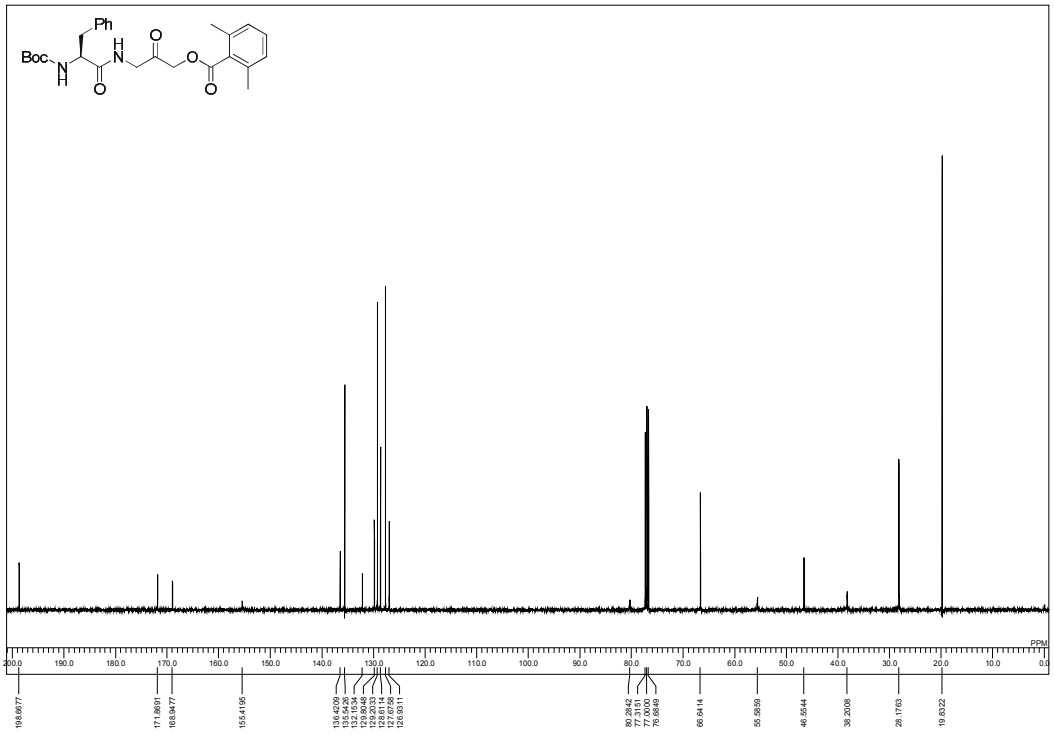
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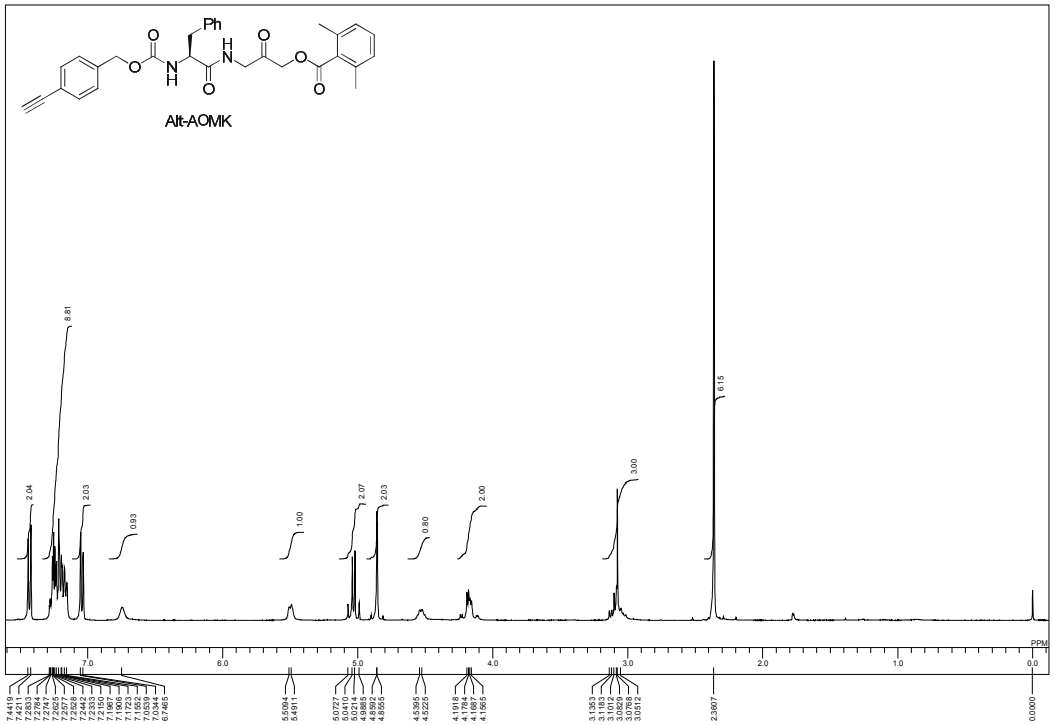
Alt-AOMK
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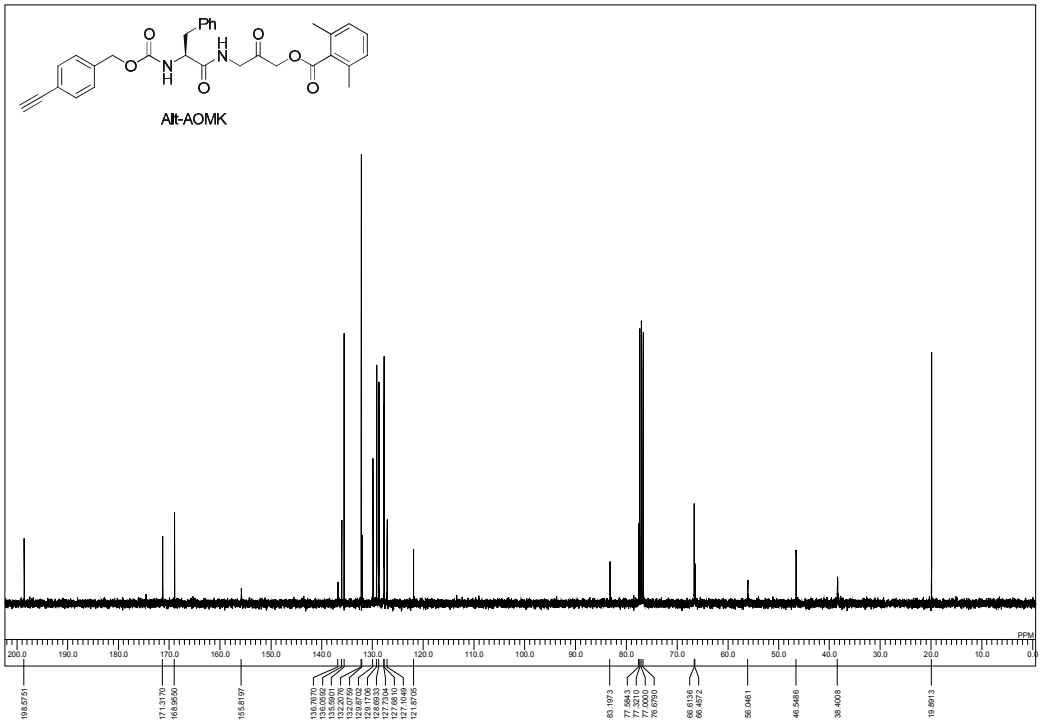
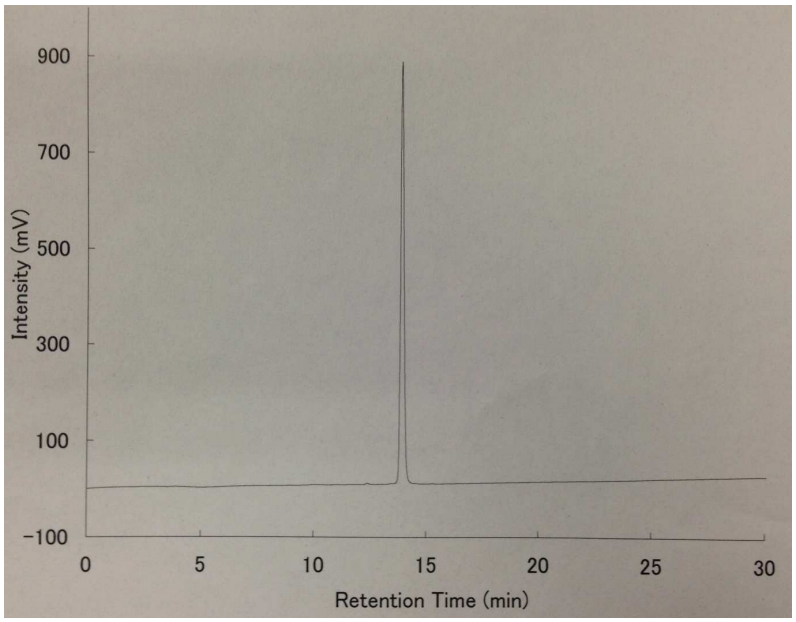
6.15
PPM

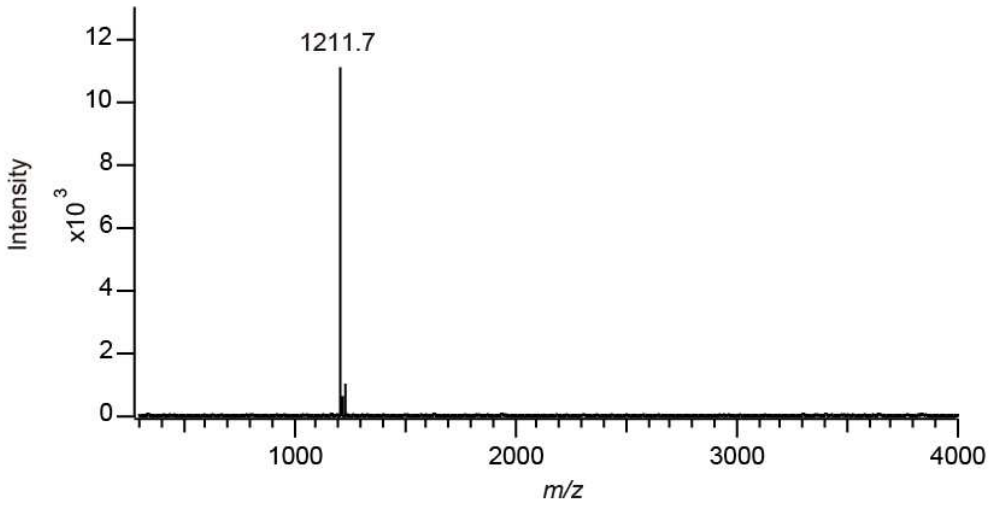

Alt-AOMK
PPM

HPLC Chromatogram and MALDI TOF-MS Spectra of Synthetic Peptides

HPLC chromatogram of synthetic peptide EQWPQCPTXK (X = propargyl glycine, alt-pept)

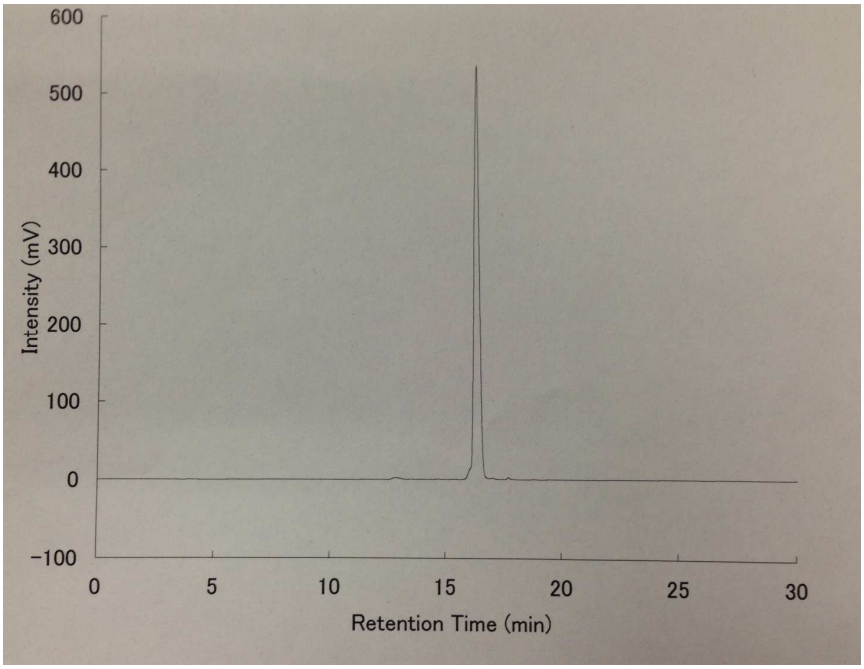


MALDI TOF-MS spectrum of synthetic peptide EQWPQCPTXK (X = propargyl glycine, alt-pept)

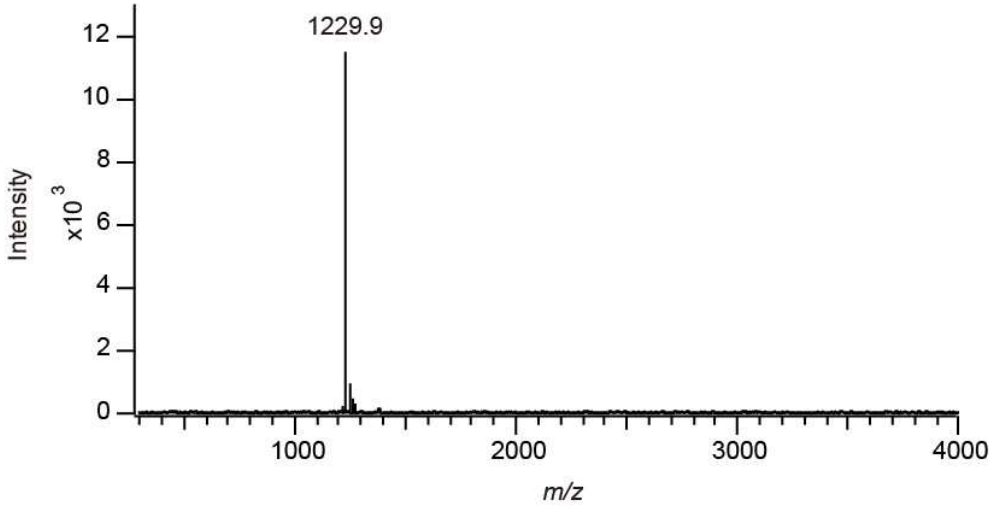


Found: *m/z* [M+H]+	1211.7
Calcd. for $C_{54}H_{79}N_{14}O_{16}S$ [M+H]+	1211.6

HPLC chromatogram of synthetic peptide EQWPQCPTIK (pept)



MALDI TOF-MS spectrum of synthetic peptide EQWPQCPTIK (pept)



Found: *m/z* [M+H]+ 1229.9

Calcd. for $C_{55}H_{85}N_{14}O_{16}S$ [M+H]+ 1229.6

Supplementary References

1. Ramjeesingh, M.; Li, C.; She, Y.-M.; Bear, C. E. *Biochem. J.* **2006**, *396*, 449-460.

2. Speers, A. E.; Wu, C. C. Bottom-up mass spectrometry analysis of integral membrane protein structure and topology. In *Comprehensive Analytical Chemistry*; Whitelegge J.; Eds.; Elsevier: Amsterdam, 2008; Vol. 52, pp 213-243.