Electronic Supplementary information for

Cell Type-Selective Imaging and Profiling of Newly Synthesized Proteomes by Using Puromycin Analogues

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1. General Information

All chemicals were purchased from commercial vendors and used without further purification. Bio-grade reagents used in this study are purchased as follows: puromycin (AG Scientific), puromycin aminonucleoside (Sigma), Tris(2-carboxyethyl)phosphine (TCEP, Sigma), Tris(3-hydroxypropyltriazolylmethyl)amine (THPTA, Sigma). All the fluorescent organelle stains were purchased from Invitrogen™. Click intermediates were purchased from Click Chemistry Tools. Reaction progress was monitored by TLC on pre-coated silica plates (Merck 60 F254, 250 μm thickness) and spots were visualized by UV light. Flash column chromatography was carried out using Merck 60 F254, 0.040-0.063 μm silica gel. ^{1}H NMR and ^{13}C NMR spectra were recorded on a Bruker 300, 400 and 500 MHz NMR spectrometer. Chemical shifts are reported in parts per million (ppm) referenced with respect to residual solvent ($CDCl_3$ = 7.26 ppm, 77.00 ppm and DMSO-d_6 = 2.50 ppm, 39.50 ppm). ^{1}H NMR coupling constants (*J*) are reported in Hertz (Hz) and multiplicity is indicated as follows: s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet). Analytical HPLC was carried out on Shimadzu LC-IT-TOF systems equipped with an autosampler, using reverse-phase Phenomenex Luna 5 μm C18 100 Å 50 × 3.0 mm columns. NIH3T3, HeLa, MCF-7, A549, A431 and CHO-K1 cells were maintained in high-glucose DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin (P/S). All cell lines were grown at 37 ºC in a humidified 5% CO_2 atmosphere. For SILAC experiments, each cell line was maintained in SILAC DMEM (Thermo), which lacks L-lysine and L-arginine, and supplemented with 10% (v/v) dialyzed FBS (Thermo), P/S (*as above*), and either [$^{13}C_6$, $^{15}N_2$]- L-lysine and [$^{13}C_6$, $^{15}N_4$]- L-arginine (Cambridge Isotope laboratories Inc) (100 μg/mL each) or L-lysine•HCl and L-arginine•HCl (Sigma) (100 μg/mL each). Heavy and light cells were maintained in parallel and passaged at least six times in isotope-containing medium before use in experiments.

2. Experimental Section

2.1 Chemical Synthesis.

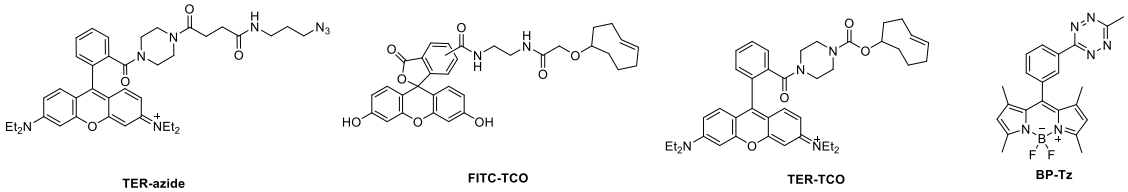
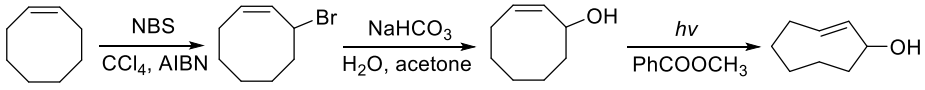
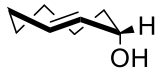


Figure S1. Chemical structure of fluorescent reporter probes used in the experiments.

Synthesis of (*E*)-cyclooct-2-enol

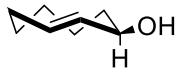


Syntheses of (*E*)-cyclooct-2-enol was based on reported literature.[1] The desired product was obtained as colorless oil. Only axial isomer was used for subsequent synthesis.



Axial Isomer.

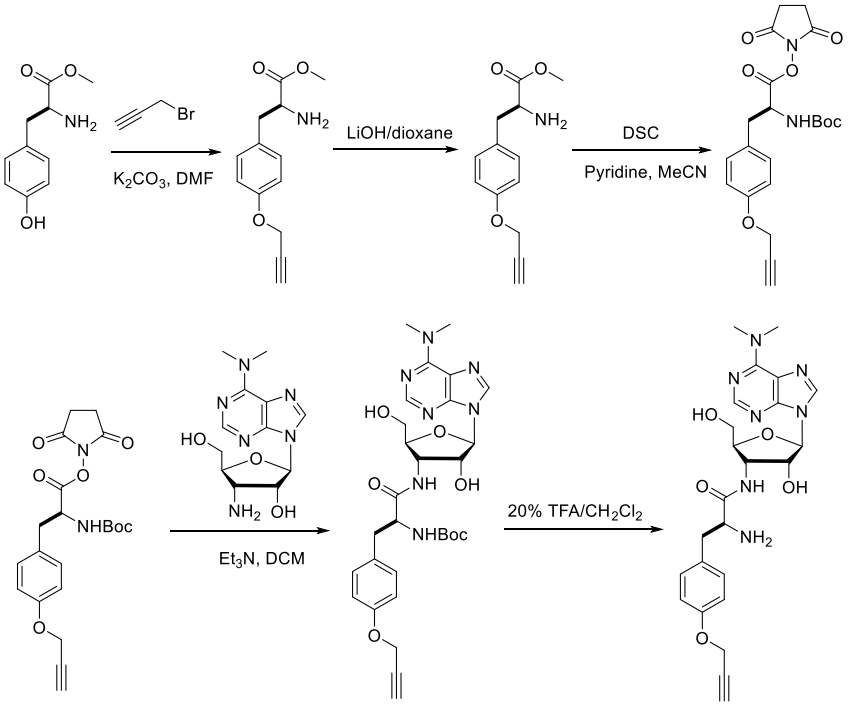
^{1}H NMR (300 MHz, Chloroform-*d*) δ 5.94 (dddd, *J* = 16.2, 11.0, 3.7, 1.1 Hz, 1H), 5.55 (dd, *J* = 16.5, 2.4 Hz, 1H), 4.63 – 4.52 (m, 1H), 2.46 (dtd, *J* = 9.8, 4.0, 1.9 Hz, 1H), 2.09 – 1.74 (m, 5H), 1.72 – 1.34 (m, 3H), 1.09 (dddt, *J* = 14.8, 12.8, 6.0, 1.7 Hz, 1H), 0.82 – 0.66 (m, 1H).



Equatorial Isomer.

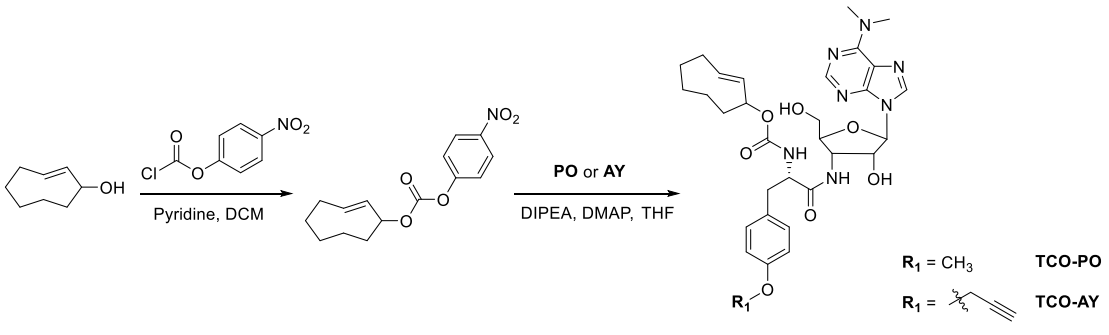
^{1}H NMR (300 MHz, Chloroform-*d*) δ 5.85 – 5.33 (m, 2H), 4.23 (ddd, *J* = 9.8, 8.6, 5.5 Hz, 1H), 2.53 (s, 1H), 2.49 – 2.29 (m, 1H), 2.27 – 2.05 (m, 1H), 2.08 – 1.65 (m, 4H), 1.65 – 1.27 (m, 2H), 1.05 – 0.54 (m, 2H).

Synthesis of (*S*)-2-Amino-N-((2S,3S,4R,5R)-5-(6-(dimethylamino)-9H-purin-9-yl)-4-hydroxy-2-(hydroxymethyl)tetrahydrofuran-3-yl)-3-(4-(prop-2-yn-1-yloxy)phenyl)propanamide (AY).



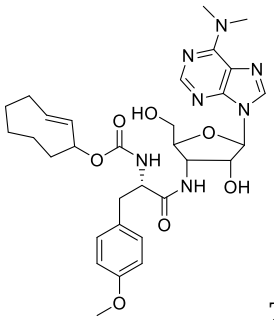
Syntheses of **AY** was based on reported literature.[2,3] **AY** was obtained as white solid. ^{1}H NMR (400 MHz, Methanol-d_4) δ 8.32 (s, 1H), 8.19 (s, 1H), 7.20 (d, *J* = 8.4 Hz, 2H), 6.97 (d, *J* = 7.0 Hz, 2H), 5.92 (d, *J* = 2.3 Hz, 1H), 4.71 (d, *J* = 2.4 Hz, 2H), 4.59 (q, *J* = 5.4, 4.7 Hz, 2H), 4.01 – 3.91 (m, 1H), 3.82 (dd, *J* = 12.5, 2.2 Hz, 2H), 3.55 (dd, *J* = 12.6, 3.0 Hz, 1H), 3.49 (s, 6H), 3.04 – 2.88 (m, 3H).

General Procedure for Synthesis of TCO-PO and TCO-AY.



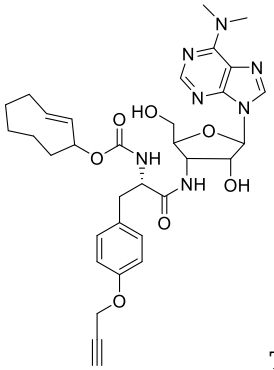
Compound **TCO-PO** and **TCO-AY** were synthesized through coupling of (*E*)-cyclooct-2-enyl (4-nitrophenyl) carbonate with puromycin or its analog based on literature.[1] Axial isomer of (*E*)-cyclooct-

2-enol (63 mg，0.5 mmol) and pyridine (60 μL, 0.75 mmol) was dissolved in anhydrous tetrahydrofuran (THF) (5 mL) and cooled at 0 °C. 4-nitrophenyl chloroformate (121 mg, 0.6 mmol) was added in multiple portions and the reaction was allowed to reach room temperature. The mixture was stirred in the dark for 4 h and the solvent was removed. THF was vaporized under reduced pressure and the reaction mixture was purified directly by column chromatography using 50:1 to 20:1 hexane and ethyl acetate to give (*E*)-cyclooct-2-enyl (4-nitrophenyl) carbonate as a light yellow solid. **PO** or **AY** (0.05 mmol, 1 eq) and DIPEA (11 μL, 0.065 mmol, 1.3 eq) was dissolved in THF (1 mL) and was cooled at 0 °C. (*E*)-cyclooct-2-enyl (4-nitrophenyl) carbonate (16 mg, 0.055 mmol, 1.1 eq) was dissolved in THF and added dropwise. Subsequently 4-dimethylaminopyridine (12 mg, 0.1 mmol, 2 eq) was dissolved in THF and added dropwise. The mixture was allowed to warm to room temperature and stirred in the dark overnight. THF was vaporized under reduced pressure and the resulting mixture was purified by column chromatography using 2:1 hexane and ethyl acetate followed by 100:2 to 100:3 dichloromethane and methanol to obtain the desired product.



TCO-PO

TCO-PO was obtained as a white solid (21 mg, 67%). ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.44 (s, 1H), 8.23 (s, 1H), 8.08 (dd, *J* = 14.0, 7.6 Hz, 1H), 7.28 (t, *J* = 9.2 Hz, 1H), 7.21 (dd, *J* = 8.3, 5.6 Hz, 2H), 6.84 – 6.79 (m, 2H), 6.09 (dd, *J* = 13.1, 4.7 Hz, 1H), 5.99 (d, *J* = 2.8 Hz, 1H), 5.82 – 5.52 (m, 1H), 5.46 (ddd, *J* = 22.0, 16.5, 2.5 Hz, 1H), 5.17 (q, *J* = 5.1 Hz, 1H), 5.05 (s, 1H), 4.53 – 4.43 (m, 2H), 4.28 (d, *J* = 12.0 Hz, 1H), 3.96 – 3.89 (m, 1H), 3.71 (s, 3H), 3.59 – 3.39 (m, 6H), 2.91 (dt, *J* = 13.5, 4.3 Hz, 1H), 2.71 (td, *J* = 12.1, 11.0, 5.8 Hz, 1H), 2.36 (t, *J* = 11.9 Hz, 1H), 1.90 (q, *J* = 19.1, 15.3 Hz, 2H), 1.76 (d, *J* = 14.2 Hz, 2H), 1.64 – 1.51 (m, 2H), 1.46 – 1.31 (m, 2H), 1.01 (d, *J* = 13.5 Hz, 1H), 0.81 – 0.71 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.96, 157.76, 155.01, 154.27, 151.83, 149.66, 137.86, 131.95, 131.91, 130.89, 130.27, 119.61, 113.38, 89.35, 83.39, 73.04, 72.85, 60.84, 56.11, 54.93, 50.29, 37.08, 35.59, 35.24, 28.40, 23.63. ESI-MS: m/z $[M+H]^+$ calcd: 624.71, found: 624.45.



TCO-AY

TCO-AY was obtained as a white solid (10 mg, 31%).^{1}H NMR (500 MHz, DMSO-d_6) δ 8.44 (s, 1H), 8.23 (s, 1H), 8.09 (dd, *J* = 13.8, 7.5 Hz, 1H), 7.30 (t, *J* = 8.5 Hz, 1H), 7.22 (dd, *J* = 8.6, 3.9 Hz, 2H), 6.90 – 6.83 (m, 2H), 6.09 (dd, *J* = 15.8, 4.7 Hz, 1H), 5.99 (t, *J* = 2.5 Hz, 1H), 5.83 – 5.70 (m, 1H), 5.63 – 5.54 (m, 1H), 5.52 – 5.41 (m, 1H), 5.17 (q, *J* = 5.1 Hz, 1H), 5.06 (s, 1H), 4.73 (dd, *J* = 4.1, 2.4 Hz, 2H), 4.47

(dd, *J* = 13.0, 6.1 Hz, 2H), 4.28 (s, 1H), 3.93 (s, 1H), 3.73 – 3.60 (m, 2H), 3.59 – 3.39 (m, 7H), 2.92 (dt, *J* = 13.6, 4.8 Hz, 1H), 2.72 (ddd, *J* = 14.0, 9.9, 5.6 Hz, 1H), 2.37 (s, 1H), 1.92 (dd, *J* = 18.3, 9.9 Hz, 2H), 1.76 (d, *J* = 11.9 Hz, 2H), 1.57 (dd, *J* = 26.2, 13.0 Hz, 3H), 1.44 – 1.31 (m, 2H), 1.01 (d, *J* = 12.4 Hz, 1H), 0.81 – 0.71 (m, 1H). ^{13}C NMR (126 MHz, DMSO-d_6) δ 171.94, 155.80, 155.08, 154.27, 151.84, 149.66, 137.86, 131.96, 131.90, 130.89, 130.25, 130.23, 119.61, 114.31, 89.34, 83.39, 79.39, 78.01, 73.05, 72.92, 60.90, 56.18, 55.33, 50.29, 37.01, 35.56, 35.24, 28.40, 23.62. ESI-MS: m/z $[M+H]^+$ calcd: 648.74, found: 648.40.

2.2. Biology

Cell Culture. NIH3T3, HeLa, MCF-7, A549, A431 and CHO-K1 cells were maintained in high-glucose DMEM supplemented with 10% (v/v) fetal bovine serum (FBS) and 100 units/mL penicillin/streptomycin (P/S). All cell lines were grown at 37 ºC in a humidified 5% CO_2 atmosphere. For SILAC experiments, each cell line was maintained in SILAC DMEM (Thermo), which lack *L*-lysine and *L*-arginine, and supplemented with 10% (v/v) dialyzed FBS (Thermo), P/S (*as above*), and either [$^{13}C_6$, $^{15}N_2$]- *L*-lysine and [$^{13}C_6$, $^{15}N_4$]- *L*-arginine (Cambridge Isotope laboratories Inc) (100 μg/mL each) or *L*-lysine•HCl and *L*-arginine•HCl (Sigma) (100 μg/mL each). Heavy and light cells were maintained in parallel and passaged at least six times in isotope-containing medium before use in experiments.

Antibody Modifications. Cetuximab (Merck 5 mg/mL) was purchased and modified with 4-(6-methyl-1,2,4,5-tetrazin-3-yl)benzoic acid that was synthesized as previously reported by our group.[4] 0.5 mg of antibody was buffer exchanged into 0.1M $NaHCO_3$ (pH 8.5) using centrifuge filtration with 10kDa molecular weight cutoff filters (Amicon) to a final concentration of 1 mg/mL. To this antibody solution, 100 equiv. of tetrazine-NHS was added. The reaction mixture was vortexed and reacted overnight at 4 ºC. After overnight reaction the antibody was purified with illustra NAP-5 column (GE Healthcare) and stored in PBS at 4 ºC. Unmodified and Tz-loaded Cetuximab (50 ng each) treated with TER-TCO (structure was shown in **Figure S1**) was resolved on 12% SDS-PAGE gel. Antibody modification was confirmed with in-gel fluorescence and silver stain (**Figure S2**).

Western Blotting. Puromycin incorporation was assessed by Western blotting (WB) with *anti*-puromycin antibody, as described in detail below. Where applicable, total protein was detected through Ponceau S staining and beta-tubulin was run as loading controls. All incorporation tests were performed in 12-well cell culture plate (Greiner). After cells were grown to 50% confluency and prepared by washing twice with PBS, 500 μL growth medium containing unmodified or **Cetuximab-Tz** at an indicated concentration was added into each well (For Cetuximab competitive experiments, cells were pre-treated with unmodified Cetuximab at indicated concentration 30 min). After 30-min incubation time, antibody solution was aspirated and cells were washed with PBS twice. **TCO-PO** was then added in 500 μL growth medium and incubated for indicated time period. The final DMSO concentration is < 0.1%. Upon completion cells were washed with PBS twice and harvested and lysed in 200 μL PBST lysis buffer (0.1% Triton, 50 μM PMSF). Protein was purified through acetone precipitation and resuspended in 1× standard SDS loading buffer, sonicated for 10 min and heated for 10 min at 95°C. Samples were then resolved on 12% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 3% non-fat milk in TBST (0.1% Tween-20) for 1h at room temperature. Anti-puromycin antibody (1:10,000 dilutions, Merck Millipore) was incubated for 1 h at room temperature, followed by washing (5×) with TBST. Then HRP-conjugated anti-mouse (Santa Cruz) was added with further incubation for 1 h at room temperature. The blot was developed by using Dura Extended Duration Substrate (Thermo Scientific) and recorded using GE ImageQuant LAS 500.

In-Gel Fluorescence Scanning/Proteome Profiling. The cell growth and incubation were the same as above. After incubation, cells were harvested and lysed in 200 μL PBST buffer (0.1% Triton, 50 μM PMSF). Click labelling was subsequently carried out with freshly premixed click reagents (10 μM **TER-N_3**, 40 μM THPTA, 400 μM TCEP and 400 μM $CuSO_4$). The reaction was incubated at room temperature for 2 h with gentle shaking before termination by addition of a five-fold volume of pre-chilled acetone (incubation for overnight at -20 °C). Precipitated proteins were subsequently collected by centrifugation (16, 000 g x 20 min at 4 °C). The supernatant was discarded and the residue pellet was washed with prechilled methanol, collected by centrifugation and finally air-dried until the pellet started to shrink. Then the residue was resuspended in 1 × standard SDS loading buffer, sonicated for 10 min and heated for 10 min at 95°C. Finally, the protein sample was separated on 12% SDS-PAGE gels followed by in-gel fluorescence scanning with a Typhoon 9410 variable mode imaging scanner (GE Amersham).

Cell Proliferation Assay. Around 5000 A431 cells/well were seeded into 96-well plates. Antibody and probe incubation was the same as described above. After three days of incubation, XTT assay was performed as described below: 50 μL of a XTT (1 mg/mL)/PMS (25 μM) solution was added per 100 μL of the medium. After incubation at 37 °C in a humidified atmosphere with 5% CO_2 for 4 h, OD value was determined on a BioTek reader using a sample wavelength of 450 nm and a reference wavelength of 650 nm. IC50 was calculated by using GraphPad Prism software.

*General Procedure for Cell Labelling and Detection by **Cetuximab-Tz**.* A431 or CHO-K1 cells were seeded in glass-bottom dishes (Greiner Bio-One, #627870) and maintained in a humidified atmosphere of 5% CO_2 at 37 °C for 24 h before being processed for imaging. After cells were grown to 50% confluency and rinsed twice with PBS, 500 μL growth medium containing Tz-loaded Cetuximab at indicated concentration was added into each well. After 30-min incubation time, antibody solution was aspirated and cells were washed with PBS twice. Cells were then incubated with **FITC-TCO** (10 μM) for 30 min, and washed twice with PBS. Then cells were co-stained with Hoechst 33342 (0.3 μg/mL, Life technologies H1399) for 15 min at room temperature. Cell labelling with **Cetuximab-Tz** was detected by confocal microscopy. All images were acquired on Leica TCS SP5X Confocal Microscope System equipped with Leica HCX PL APO 63x/1.20 W CORR CS, 405 nm Diode laser, Argon ion laser, white laser (470 nm to 670 nm, with 1 nm increments, with 8 channels AOTF for simultaneous control of 8 laser lines, each excitation wavelength provides 1.5 mV), an PMT detector ranging from 420 nm to 700 nm for steady state fluorescence. Images were processed with Leica Application Suite Advanced Fluorescence (LAS AF).

General Procedure for Immunofluorescence Imaging of Puromycin. A431 or CHO-K1 cells were seeded in glass-bottom dishes (Greiner Bio-One, #627870) and grown for 24 h. After cells were grown to 50% confluency and rinsed twice with PBS, 500 μL growth medium containing **Cetuximab-Tz** (10 μg/mL) was added into each well. After 30-min incubation time, antibody solution was aspirated and cells were washed with PBS twice. **TCO-PO** was then added in 500 μL growth medium and incubated for indicated time period. The final DMSO concentration is < 0.1%. Upon completion cells were washed with PBS twice. Subsequently, cells were fixed with cold methanol for 15 min at -20 °C and washed with PBS twice. The cells were blocked with 2% BSA in PBS for 30 min at room temperature and washed with PBS twice.

For immunofluorescence (IF), cells were incubated with *anti*-puromycin (1:2000) in PBS (2% BSA) overnight with gentle agitation at 4 °C and washed with PBS (3×2 min). Then secondary antibody *anti*-mouse IgG-HRP (diluted to 1:100 in 2% BSA in PBS) was added and incubation continued for another 1 h with gentle agitation before washing with PBS (3×2 min). An Alexa Fluor® 488 labelled tyramide

working solution was freshly prepared by diluting the tyramide stock solution 1:100 in amplification buffer/0.0015% H_2O_2. 300 µL of the tyramide working solution was applied to each well and incubated for 10 minutes at room temperature with gentle agitation. The cells were washed three times with PBS followed by co-staining with Hoechst 33342. All images were acquired on a Leica TCS SP5X Confocal Microscope System.

Co-Culture of A431 and CHO-K1. Before experiments, cells were grown to 90% confluency, released using 0.05% Tryspin/0.53 mM EDTA, and prepared by washing twice with PBS. A431 cells were labelled with Mitotracker (18 nM, MitoTracker® Red CMXRos, M7512) in growth medium for 1 h at 37 °C. Following centrifugation and aspiration of the Mitotracker solution, A431 cells were washed twice by centrifugation with PBS. Then equal number of A431 and CHO-K1 cells were resuspended in growth medium separately, and then the two cell lines were combined together and homogenized by gently pipetting. The mixture of A431 and CHO-K1 cells were seeded in glass-bottom dishes (Greiner Bio-One, #627870) and grown for 24 h to 50% confluency, and cell labelling and immunofluorescence imaging experiments were carried out as describe above.

Transfection of EGFR-mCherry in CHO-K1 Cells. EGFR-mCherry plasmid and CHO-K1 cells were kindly provided by Prof. Thorsten Wohland (NUS, Singapore). For EGFR-mCherry transfection experiments, 0.8 µg of the plasmid and 2 µL of Lipofectamine 2000™ (Invitrogen) were used for each transfection according to the manufacturer's protocol. One day before transfection, CHO-K1 cells were seeded into 4-well imaging dish (Greiner Bio-One, #627870) with 0.5 mL/well of DMEM (containing 10% FBS but without antibiotics). The cells were left for 24 h to reach ~80% confluency. Prior to transfection cells were prepared by rinsing with DMEM (without FBS or antibiotics) twice and changed to 0.4 mL/well fresh DMEM (containing 10% FBS but without antibiotics). Lipofectamine™ 2000 (2 µL/well) was diluted in 50 µL of Opti-MEM® (ThermoFisher, 31985070) and mixed gently before incubation for 5 min at room temperature. Meanwhile EGFR-mCherry plasmid (0.8 µg/well) was diluted in 50 µL of Opti-MEM®. After the 5 minute incubation, the diluted DNA was combined with the diluted Lipofectamine™ 2000 (total volume is 100 µL) and incubated for 20 minutes at room temperature to allow the DNA-Lipofectamine™ 2000 complexes to form. The 100 µl of DNA-Lipofectamine™ 2000 complexes was added to each well containing cells and plating medium. After gently rocking the plate back and forth, the cells were maintained in a humidified atmosphere of 5% CO_2 at 37 °C for 48 h before further experiments. Cell labelling and immunofluorescence imaging experiments were carried out as describe above.

2.3 LC-MS Characterizations of Click-and-Release Reaction between TCO-PO and Tetrazine.

10 µL of **TCO-PO** (1 mM in DMSO) was mixed with 15 µL of **2** (1 mM in DMSO). The mixture was incubated for 30 min and subjected to LC-MS analysis (6.25 µL/injection). All samples were tested under the same conditions (1-100% CH_3CN/H_2O over 10 min with detection channels at 254 nm). For the ligation reaction, different regioisomers could form, but for simplicity, only one regioisomer was depicted.

2.4 Proteomics

After incubation with the probes at indication conditions, cells from 10-cm dishes were harvested and lysed by probe sonication in 1 mL DPBST buffer (0.1% Triton, 50 µM PMSF). The protein concentration was normalized to 2.5 mg/mL by BCA assay. Isotopically heavy and light proteomes were mixed in equal proportions. Freshly premixed click reagents (final concentration: 50 µM TER-Biotin-N_3,

200 μM THPTA, 1 mM TCEP and 1 mM $CuSO_4$) were added. The reaction was incubated at room temperature for 2 h with gentle shaking before being terminated by addition of a five-fold volume of pre-chilled acetone. After incubation overnight at -20 °C, the collected proteome was washed with 4:1 methanol:chloroform (1 mL×2). The final proteome was dissolved in 1 mL urea solution (6 M) and 120 μL 10% SDS solution was added to dissolve the whole proteome. Insoluble fraction was removed by centrifugation (4000 rpm, 20 min). 100 μL of NeutrAvidin™ agarose beads (Thermo Scientific) pre-equilibrated in DPBS was added to the solution and the resulting mixture was rotated at room temperature for 3 h. The beads were collected by centrifugation (1000 rpm × 1 min) and washed with DPBS (3× 10 mL), 1% SDS (3×1 mL), 3% SDS (1 mL), 1% SDS (3×1 mL), DPBS (3×1 mL) and ddH_2O (3× 1 mL). 100 μL of 2× standard SDS-PAGE loading dye was added and the sample was boiled at 95 °C for 30 min. The elution was collected as pull-down (PD) samples. The PD samples were then separated on 12% SDS-PAGE and subjected to in-gel tryptic digestion and LC-MS/MS analysis as previously reported.[3] LC-MS analysis was performed on a nanoAcquity (Waters, inc) configured with Q-exactive Orbitrap mass spectrometer (Thermo Scientific). Digests were loaded onto a fused silica desalting column packed with 2 cm C18 reverse phase resin (Phenomenex Inc.), and peptides were eluted on a reverse phase column (100 μm fused silica packed with 25 cm C18 resin) using a gradient 5-50% Buffer B in Buffer A (Buffer A: water, 0.1 % formic acid; Buffer B: acetonitrile, 0.1% formic acid). The flow rate through the column was set to 250 nl min-1and spray voltage was set to 2.0 kV. One full MS scan (350 – 1800 MW) was followed by 10 data dependent scans of most intense ions with dynamic exclusion enabled. The generated tandem MS data was searched using Uniprot (Taxonomy: Human, 2017.04.11 for A431 cells; (Taxonomy: 10029 - Cricetulus griseus, 2017.4.29) for CHO-K1 cells) using Proteome Discoverer software (v.1.4.0.288). A maximum of three missed cleavages were allowed and fully tryptic peptides were considered with dynamic modification of acetyl at N-terminal. The maximal mass tolerance in MS mode was set to 10 ppm, and fragment MS/MS tolerance was set to 0.8 Da for HCD data. The maximum peptide and site false discovery rates were specified as 0.01 using Percolator. Quantitative SILAC ratio was calculated using Proteome Discoverer "Precursor Ions Quantifier" modules. The obtained peptide/protein list for each fraction was exported to Microsoft Excel or processed using an in-house script for further analysis.

3. Results and Discussion

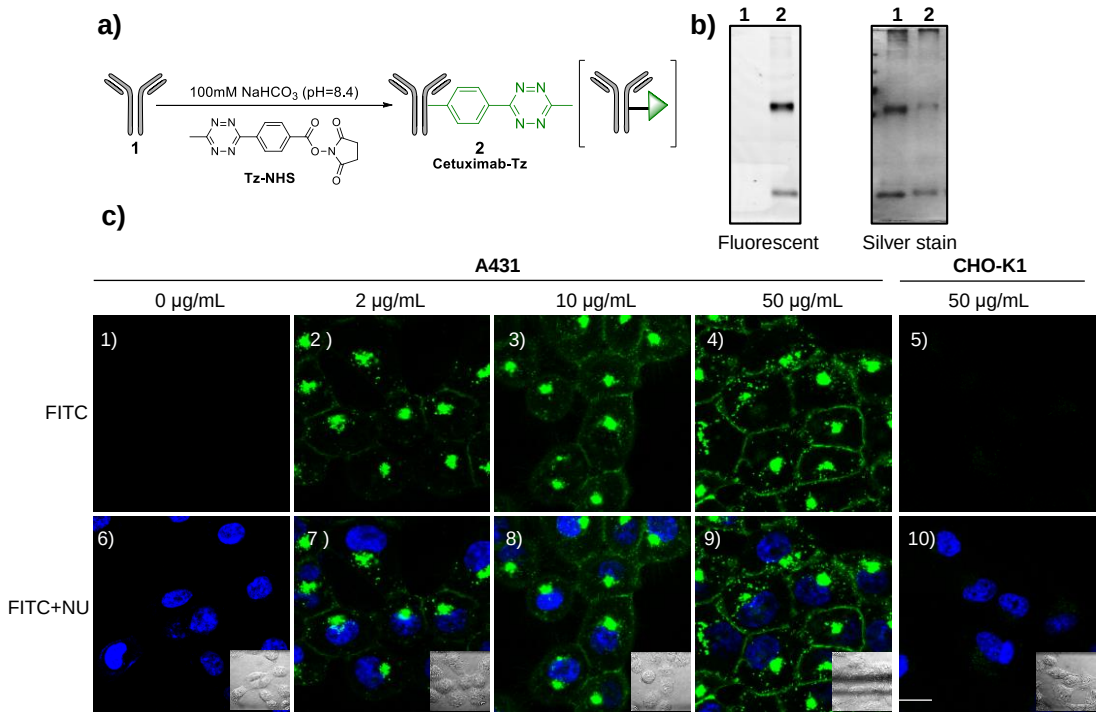


Figure S2. (a) Modification of **Cetuximab** with **Tz-NHS**. (b) Original and tetrazine-loaded **Cetuximab** were reacted with **TER-TCO** and resolved on 12% SDS-PAGE gel. (left) in-gel fluorescence, (right) silver stain. 1. Original **Cetuximab**; 2. **Cetuximab-Tz**. (c) Confocal microscopy images of antibody labelled cells. A431 cells were treated with **Cetuximab-Tz** (indicated concentration where applicable) for 30 min then **FITC-TCO** (10 µM) for 30 min. In all cases, a strong fluorescence signal was detected at the cell membranes together with internalized antibody; this was not observed for CHO-K1.

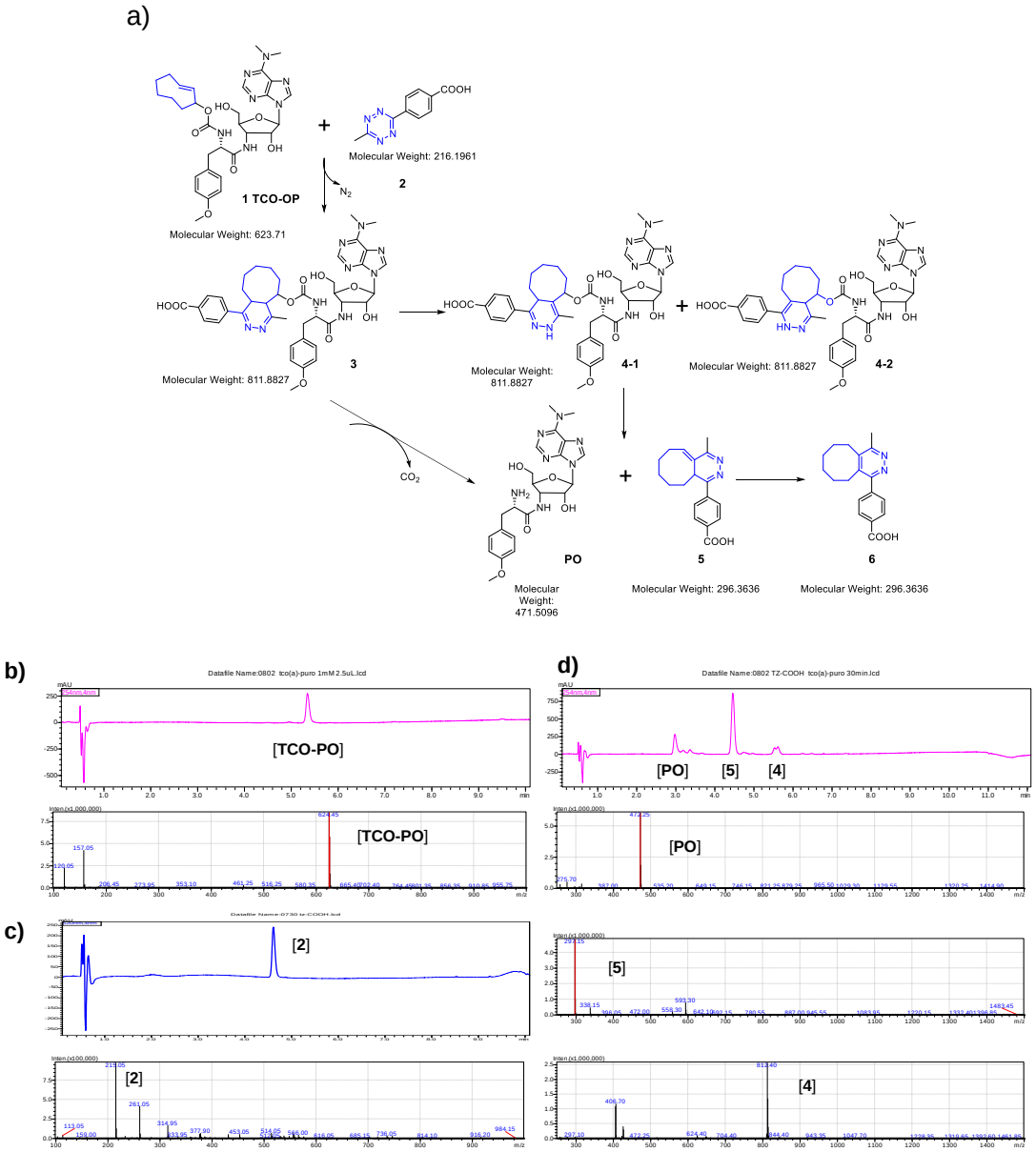


Figure S3. (a) Release mechanism from inverse-electron-demand Diels–Alder (inv-DA) product **3**, following the reaction of **TCO-PO** with tetrazine **2**; not all possible tautomer conversions and stereoisomers are shown. (b-d) LC-MS analysis of reaction between **TCO-PO** and tetrazine **2**. As indicated by LC-MS analysis, inv-DA adduct **3** was not fully converted to **PO**. The release efficiency was approximately 70% judging from the area. There is a small amount remaining as **4** (30%). However gel profiling and imaging experiments showed that the released amount was sufficient to label nascent polypeptides.

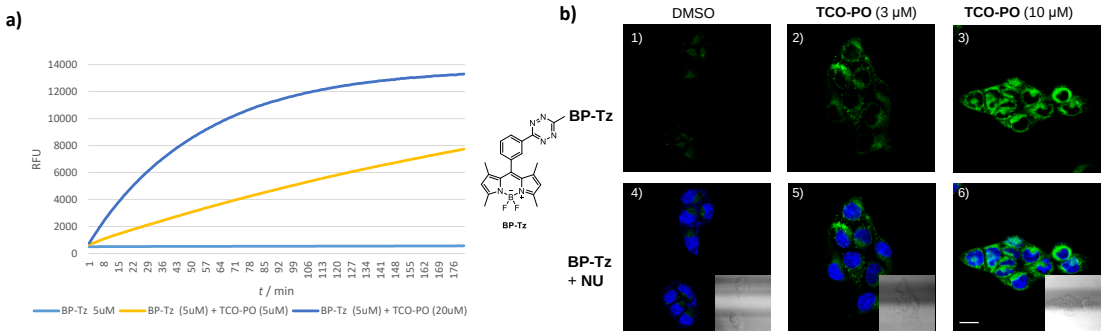


Figure S4. (a) Time-dependent fluorescence changes of 10 μM **BP-Tz**[5] with or without **TCO-PO** (5 μM, 20 μM). The fluorescence of **BP-Tz** was quenched internally due to the presence of tetrazine and will be "Turned-On" upon reaction with **TCO-PO**. (b) Cell-permeability test of **TCO-PO** through imaging experiment with **BP-Tz**. A431 cell were incubated with **TCO-PO** for 1 h (3 μM, 10 μM, where applicable). **TCO-PO** solution was aspirated and cells were washed briefly with fresh medium to remove **TCO-PO** that was not taken up by cells. Then **BP-Tz** (10 μM) was introduced and images were taken immediately. The turned-on fluorescence of BODYPY indicated the presence of **TCO-PO** inside the cells (2 & 3), whereas there was very low fluorescence in DMSO-treated cells (1).

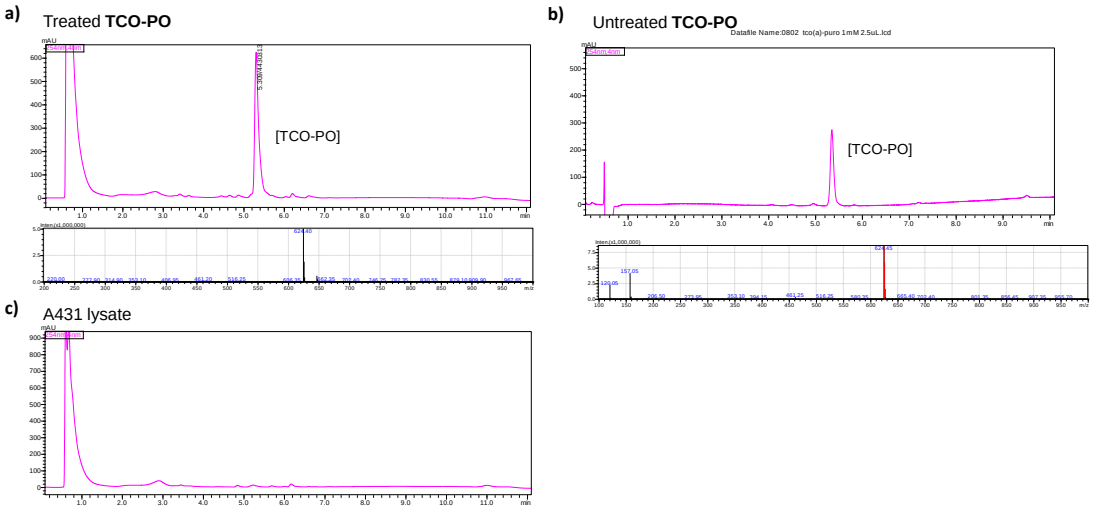


Figure S5. Hydrolytic stability test of **TCO-PO**. A431 cell lysate was prepared in PBS (with 1 mM PMSF) by probe sonication and normalized to 2 mg/mL. 1 μL of 5 mM **TCO-PO** in DMSO was added into 100 μL cell lysate. After incubation at 37 °C for 24 h, chilled acetone was added and protein was pelleted through centrifuge. Supernatant was collected, dried, redissolved in water/DMSO (1:1) and analyzed by LCMS. LCMS profiles of treated (a) and untreated **TCO-PO** (b) confirm that **TCO-PO** remains intact after 24 h, and no **PO** was observed.

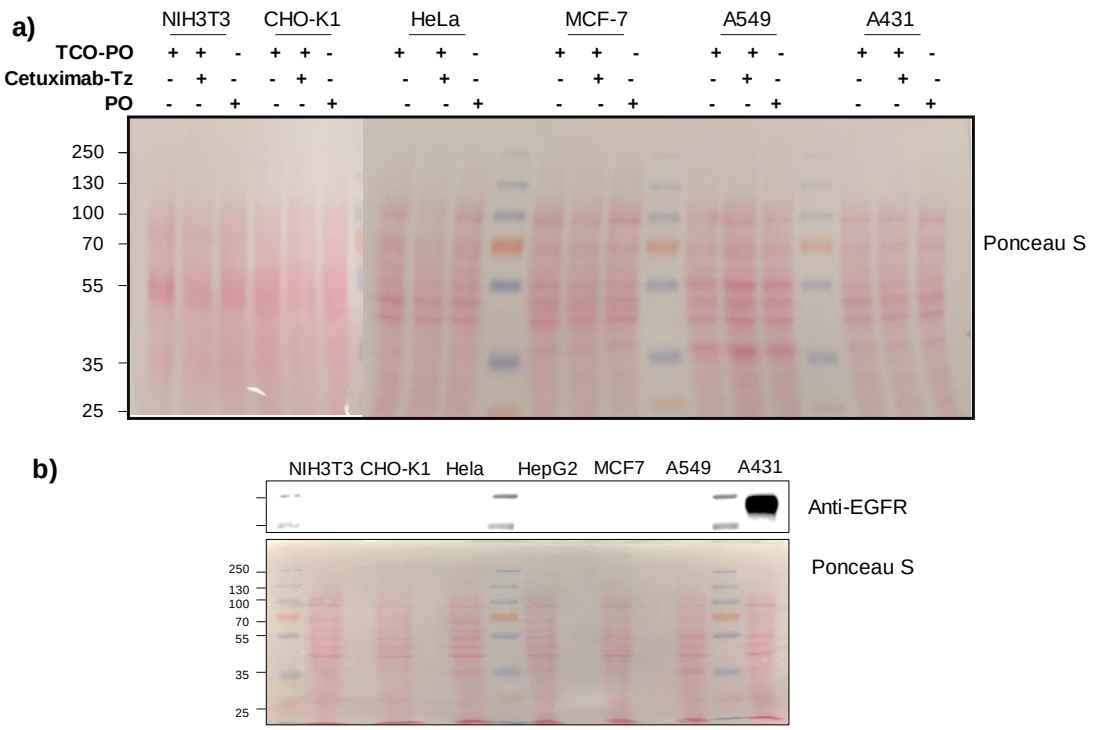


Figure S6. (a) Total proteins detected by Ponceau S stain for **Figure 1b**. (b) Levels of EGFR expression for cell lines in **Figure 1b** were monitored by *anti*-EGFR. Ponceau S stain serves as a loading control.

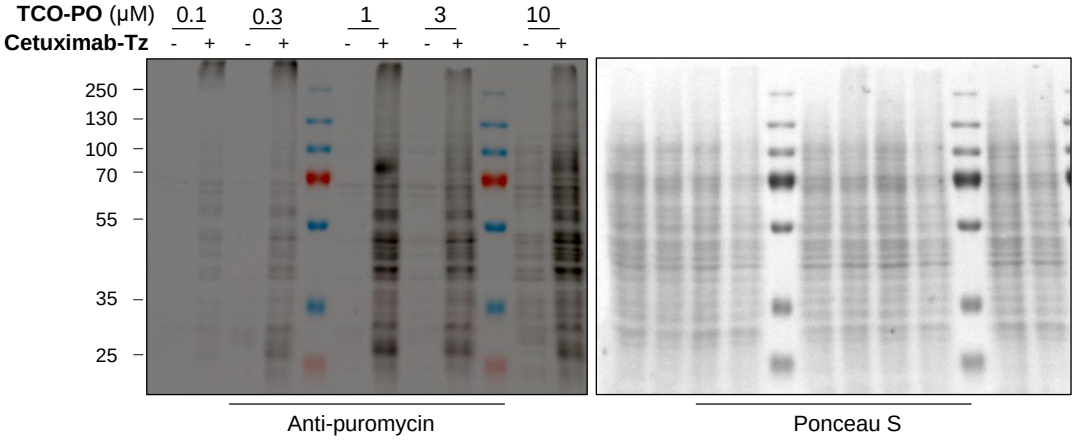


Figure S7. Concentration-dependent incorporation of **PO** in nascent polypeptides through antibody-assisted release. Nascent protein synthesis was detected by western blotting using *anti*-puromycin (left). Total protein was indicated by Ponceau S stain (right).

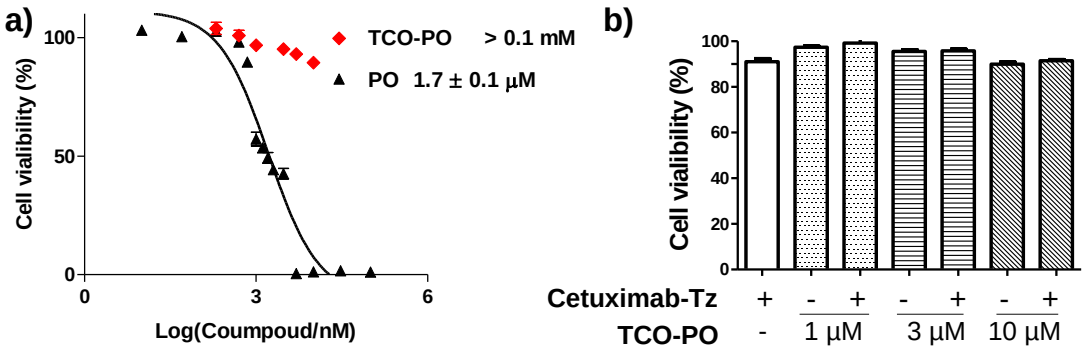


Figure S8. (a) XTT cell anti-proliferation results of **PO** and **TCO-PO** in A431 cells. The IC_{50} value for **PO** is 1.7 µM, while there is no significant cell toxicity of **TCO-PO** in working range. (b) XTT cell anti-proliferation results of **Cetuximab-Tz** and **TCO-PO** in A431 cells. The strategy showed no cell toxicity over the working range. This indicated that **PO** released was below the IC_{50} value, as a result the strategy can detect protein synthesis without altering cell's native state.

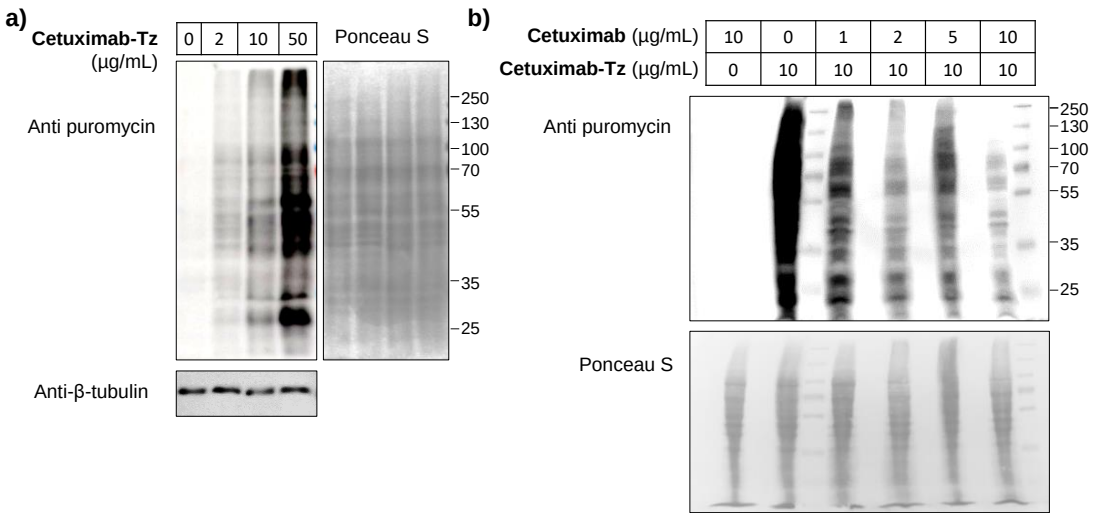


Figure S9. (a) A431 cells treated with increasing doses of **Cetuximab-Tz** for 30 min showed a significant increase in puromycin incorporation. (b) The labelling can be partially blocked by pre-incubation with unmodified **Cetuximab**. Ponceau S stain and anti β-tubulin serve as a loading control.

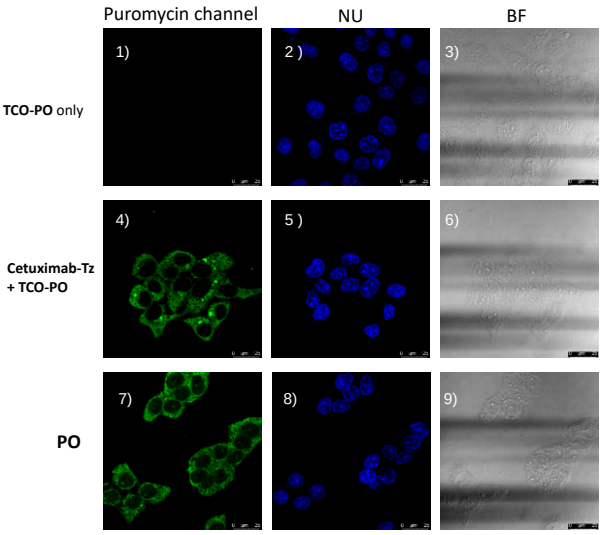


Figure S10. IF signals of A431 cells treated with **Cetuximab-Tz** followed by **TCO-PO** were shown to be uniformly distributed in cytosol, which is similar to cells treated directly with puromycin.

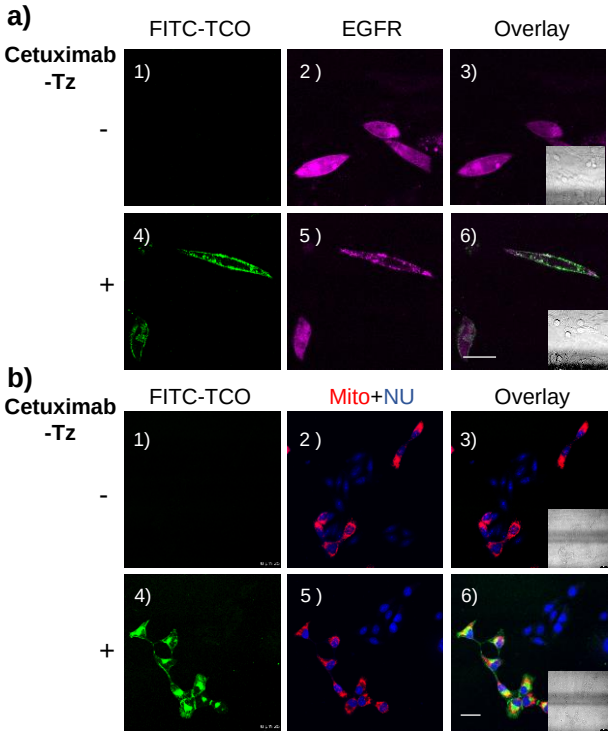


Figure S11. Selective binding of **Cetuximab-Tz** in CHO-K1 cells transfected with EGFR-mCherry (a) and in A431 cells in heterogeneous environment (b). Cells were treated with **Cetuximab-Tz** (10 µg/mL, where applicable) for 30 min then **FITC-TCO** for 24 h. Cells were fixed with cold methanol and imaging experiment with was performed. Scale bar = 25 µm.

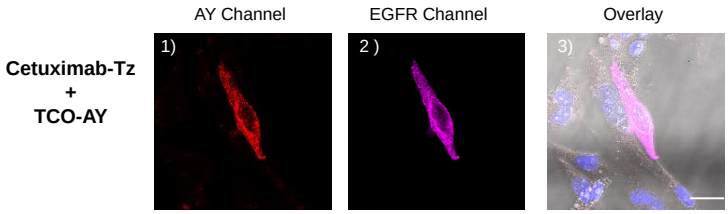


Figure S12. Selective labelling of nascent polypeptides in CHO-K1 cells transfected with EGFR-mCherry. Cells were treated with **Cetuximab-Tz** (10 μg/mL) for 30 min then **TCO-AY** for 24 h. Cells were fixed with cold methanol and click reaction with **TER-azide**. Total cells were detected by DAPI (blue).

4. Reference

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[2] J. Liu, Y. Xu, D. Stoleru and A. Salic, *Proc. Natl. Acad. Sci. USA,* 2012, **109**, 413-418.

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[4] P. Wang, Z. Na, J. Fu, C. Y. Tan, H. Zhang, S. Q. Yao and H. Sun, *Chem Commun (Camb)*, 2014, **50**, 11818-11821.

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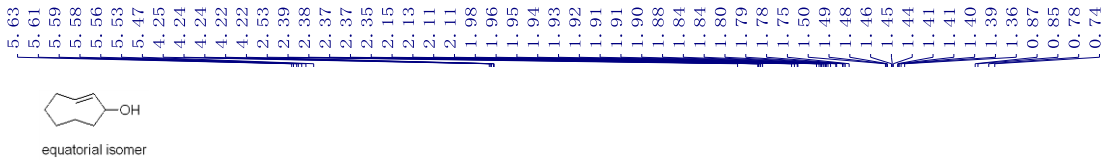
5. ^{1}H & ^{13}C NMR Spectra



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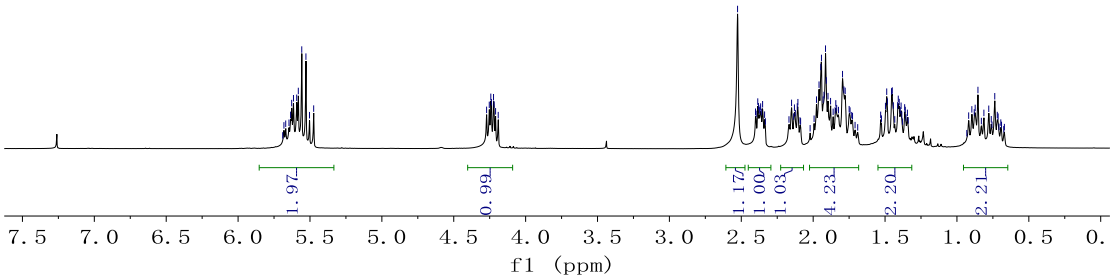

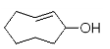

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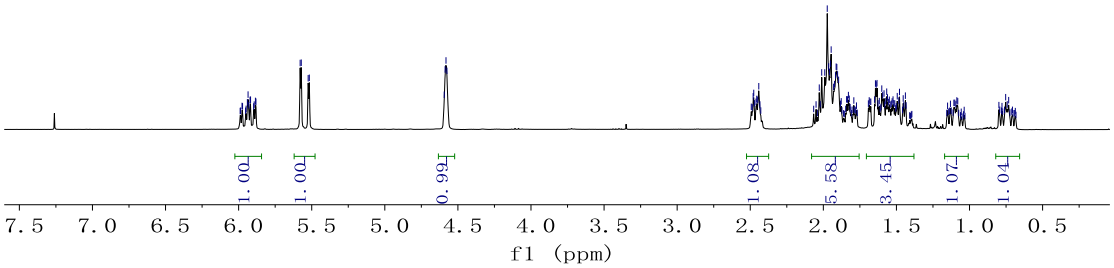

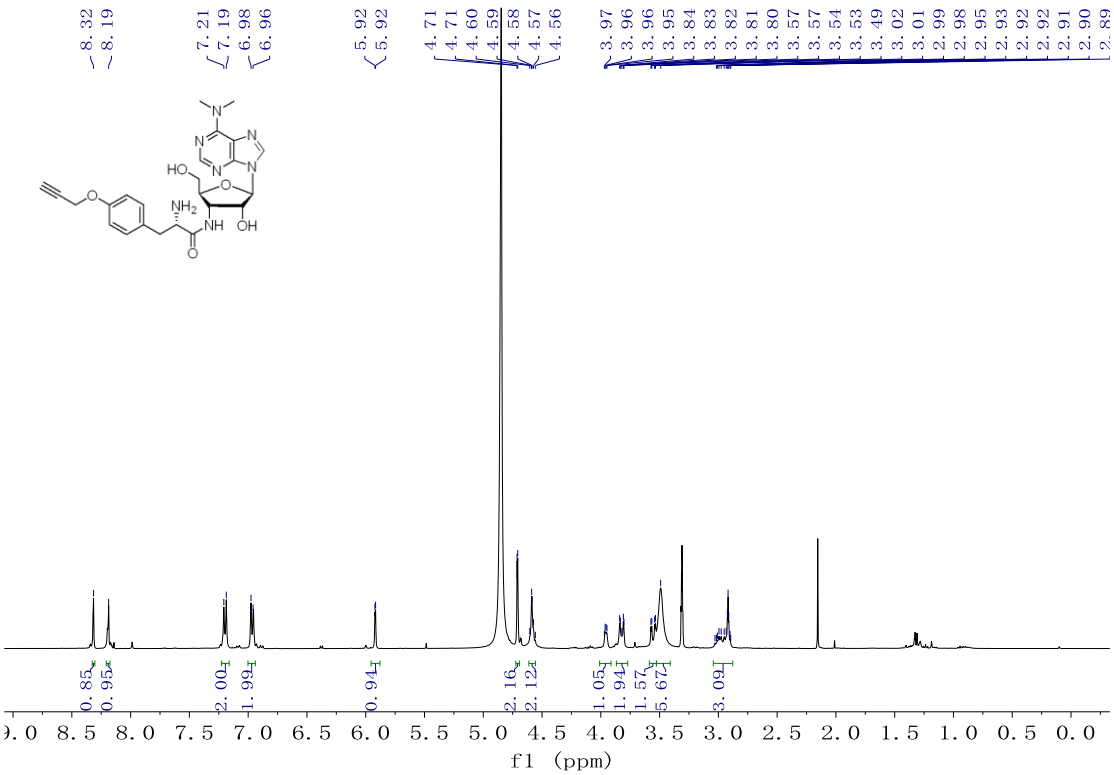
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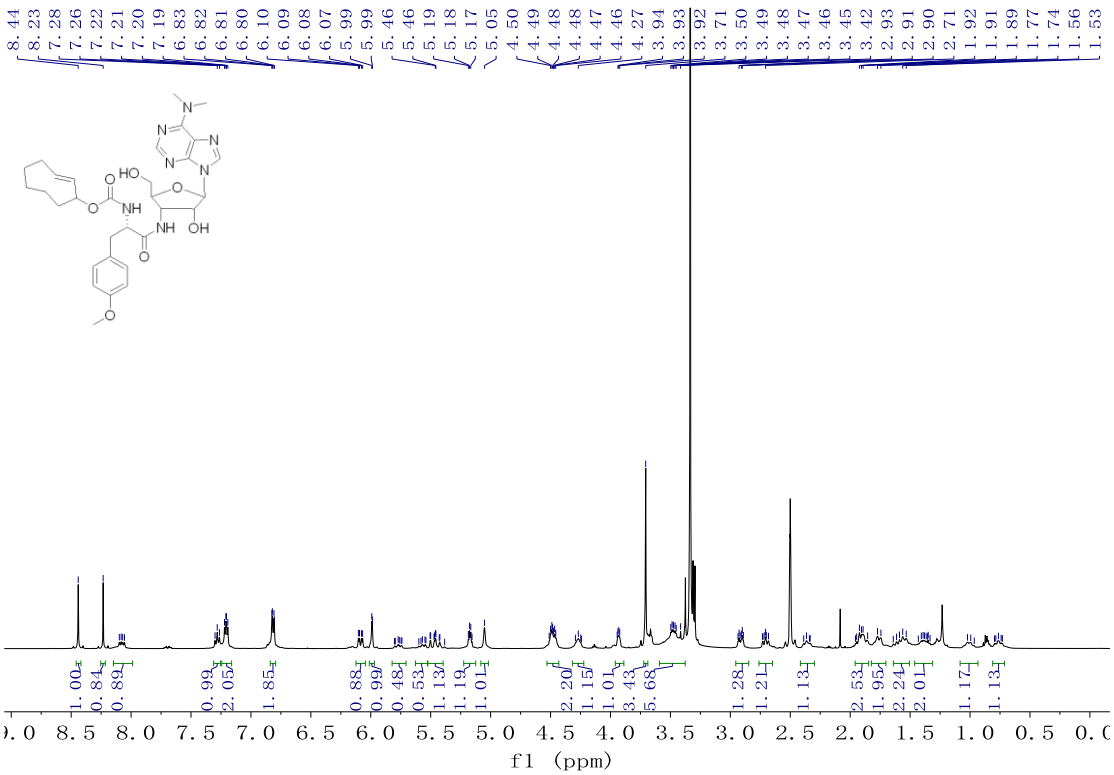
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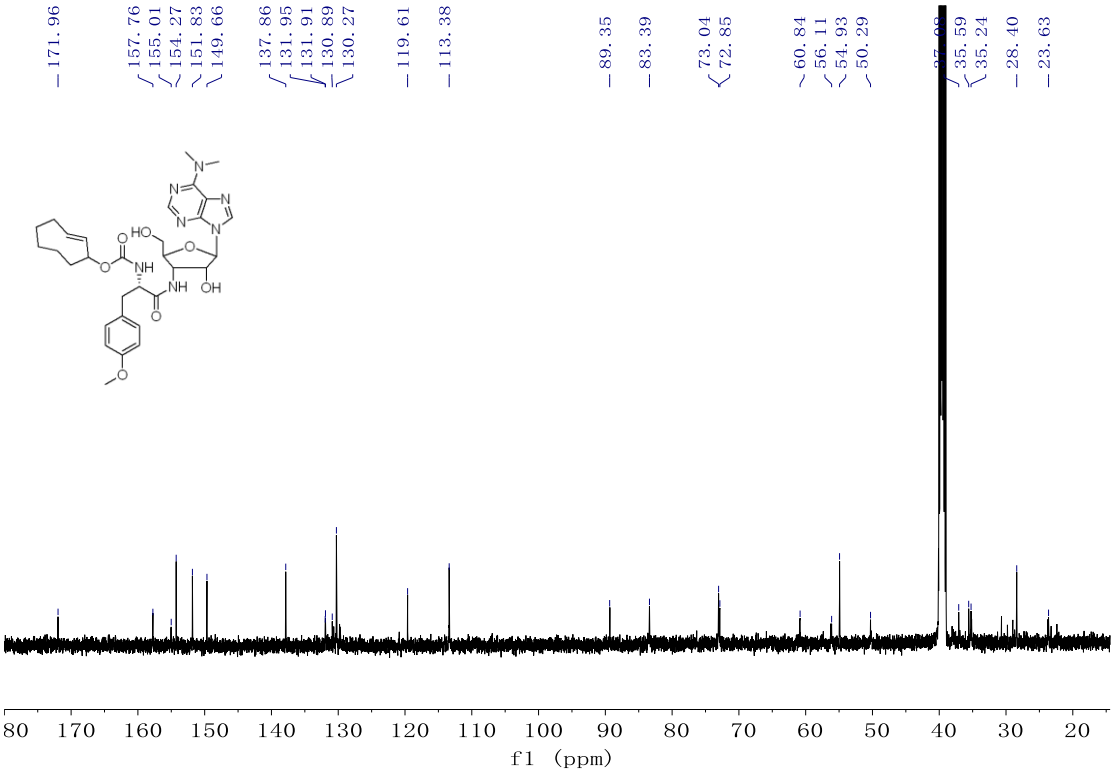
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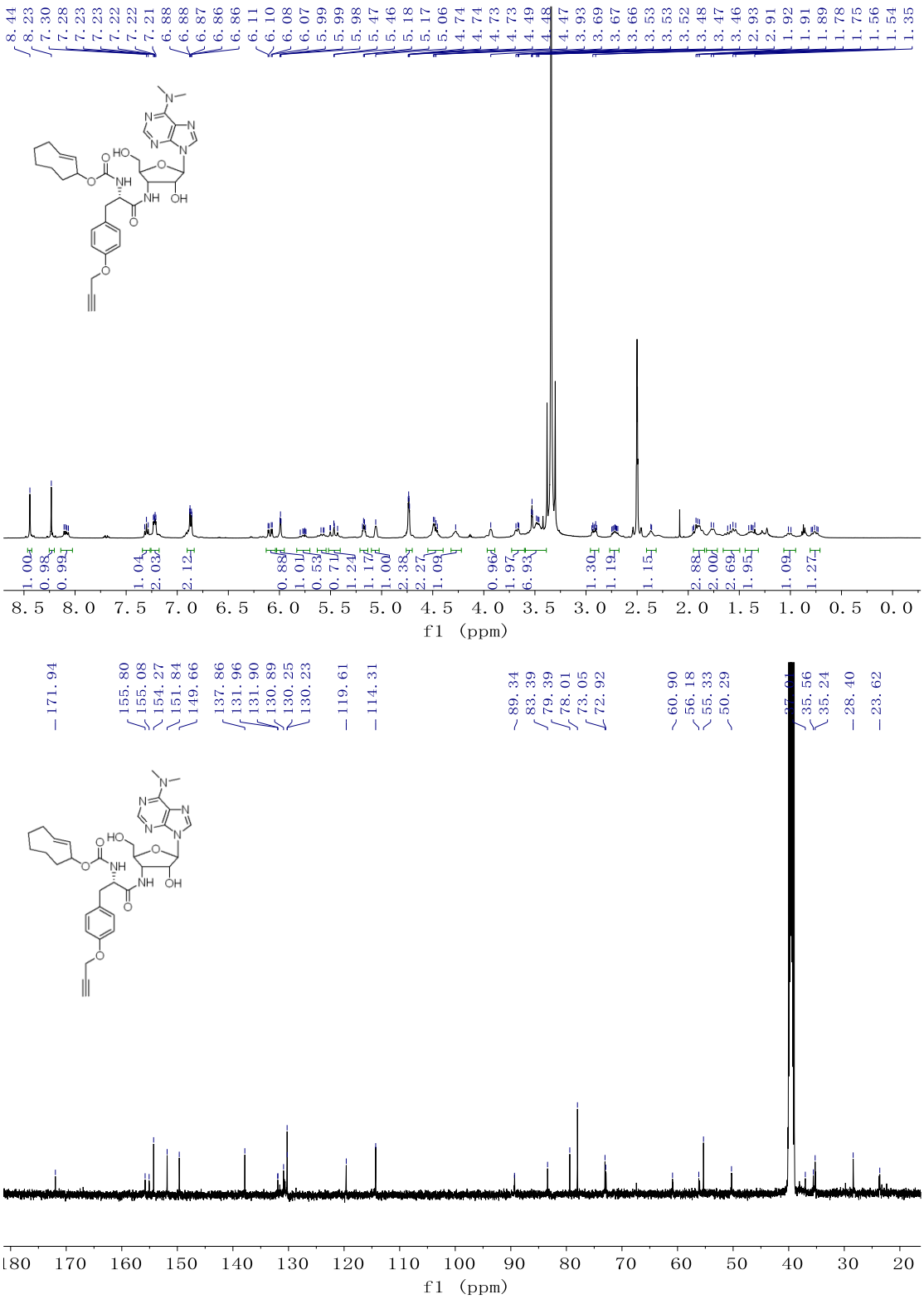
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