Electronic Supplementary Information for

A Bioorthogonal Chemistry Strategy for Probing Protein Lipidation in Live Cells

Wenjiao Song, Zhipeng Yu, Michael M. Madden, and Qing Lin*

Department of Chemistry, State University of New York at Buffalo, Buffalo, New York 14260 USA

Materials and General Procedures

Unless otherwise noted, all chemicals were obtained from commercial sources and used without further purification. Amino acid derivatives, coupling reagents, and resins were purchased from Novabiochem (San Diego, CA) and AnaSpec (San Jose, CA). pTXB1 vector and chitin beads were purchased from New England Biolabs (Ipswich, MA). Flash chromatography was performed with SiliCycle silica gel 60 Å (40-63 μm). All reactions were carried out under argon using oven-dried glassware. ^{1}H-NMR spectra were recorded on an Inova 500 MHz NMR instrument. Chemical shifts were reported in parts per million (ppm) relative to internal solvent standards. Multiplicities were reported as follows: singlet (s), doublet (d), triplet (t), doublet of doublet (dd), quintet (q) or multiplet (m). High-resolution ESI-MS analysis was performed by SUNY Buffalo Instrument Center.

Mass Spectrometry. Electrospray LC-MS analysis was performed using a Finnigan LCQ Advantage Ion Trap mass spectrometry coupled with a Surveyor HPLC system. Small molecule liquid chromatography was performed using a DIONEX Acclaim120 3 μm C_{18} 120Å reverse-phase column (2.1 × 100 mm). Protein liquid chromatography was performed using a Phenomenex Jupiter 5 μm C_5 300Å reverse phase column (2.00 × 150 mm) with a flow rate of 250 μL/min. A linear gradient of 5-90% B over 30 min was applied for all runs in which solvent A was 0.1% aqueous formic acid and solvent B was 100% acetonitrile containing 0.1% formic acid. Protein intact mass deconvolution was performed using the software BioworksBrowser (ThermoFinnigan). Prior to MS analysis, protein samples were desalted and/or separated from small molecules using gel filtration as indicated.

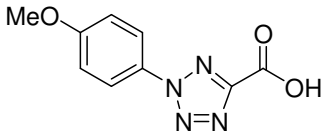
High Perfomance Liquid Chromatography. HPLC was performed on a GILSON 215 HPLC System. Analytical runs for modified peptides were performed using Keystone scientific BetaBasic-18 column (4.6 × 250 mm). Flow rate was 1.5 mL/min and UV detection was set at 220 and 254 nm. Purification of

the small-molecule modified peptides was performed using Phenomenex Luna 5 μm C_{18} 100 Å (10.00 × 250 mm) column with a flow rate of 5 mL/min. Analysis of small-molecule modified proteins was performed using GRACE Vydac 218TP54 5 μm C_{18} column (4.6×250 mm) with a flow rate of 1.5 mL/min and UV detection set at 220 and 254 nm.

Gel Analysis. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of proteins was performed on an XCell SureLock Mini-Cell apparatus using NuPAGE 4-12% Bis-Tris gels (Invitrogen). BenchMark Prestained Protein Ladder was applied to at least one lane of each gel for estimation of apparent molecular weights. Visualization of protein bands was accomplished by staining with SimplyBlue SafeStain solutions (Invitrogen). For fluorescent gel imaging, the gel was illuminated from one side using a hand-held 365 m UV lamp and the fluorescence image was recorded using a digit camera.

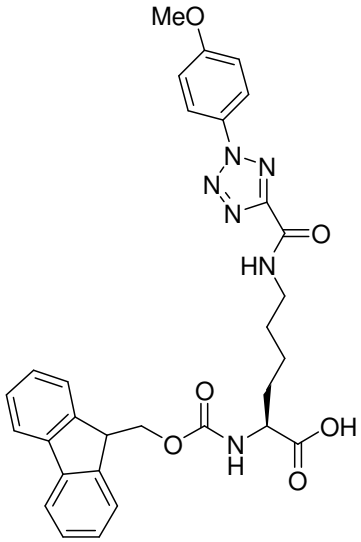
Gel Filtration. Before LC-MS and HPLC analysis, gel filtration chromatography (Sephadex G-25, GE Healthcare) was performed to remove salts and small molecules from protein samples with ddH_2O as the elution buffer. Size exclusion chromatography (Superdex 75, Amersham Biosciences) was performed to isolate the tetrazole-modified EGFP from the ligation mixture with an elution buffer of 20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 7.5.

Synthesis of Tetrazole Tails:



2-(4-Methoxy-phenyl)-2*H*-tetrazole-5-carboxylic acid: To 1.6 mL of ethyl glyoxialate in toluene was added 130 mL ethanol solution of sulfonyl hydrazide (1.38 g, 8.0 mmol). The resulting mixture was stirred for 1 hr at room temperature in an open flask before being pored over 100 g of ice. A white precipitate (sulfonyl hydrazone) was generated, which was collected by filtration. The filtrate was subsequently dissolved in 50 mL pyridine to derive solution A. Separately, to a 7.2 mmol solution of 4-methoxy-aniline in a 3:1 mixture of ethanol and water (20 mL cooled to -5 °C) was added 3.0 mL of concentrated HCl. To the resulting acidic aniline solution, 4 mL solution of sodium nitrite (0.690 g, 10 mmol) was added dropwise to generate the phenyl diazonium salt (solution B). Solution A was cooled to -20 °C and solution B (kept at -5 °C) was added dropwise to solution A over a period of 30 minutes. Following addition, the reaction mixture was allowed to warm up to room temperature over 60 minutes and subsequently extracted with ethyl acetate three times. The organic layer was then washed with 0.1 N HCl, dried over sodium sulfate, and concentrated. The crude mixture was purified via a silica gel chromatography using a stepwise gradient of ethyl acetate/hexane to afford 2-(4-methoxy-phenyl)-2*H*-tetrazole-5-ethyl ester as a light yellow-brown powder. Following purification, sodium hydroxide (7.5 mmol; 0.6 g dissolved in 2 mL of

water) was added to a 50 mL solution of 2-(4-methoxy-phenyl)-2*H*-tetrazole-5-ethyl ester in ethanol. The mixture was refluxed for 2 hrs before cooling down to room temperature. The mixture was acidified with a dilute HCl solution. The resulting 2-(4-methoxy-phenyl)-2*H*-tetrazole-5-carboxylic acid was extracted three times with ethyl acetate to yield the titled compound (0.55 g, 35%): ^{1}H NMR (500 MHz, DMSO-d_6) δ 8.02 (d, J = 9.5 Hz, 2H), 7.20 (d, J = 8.5 Hz, 2H), 3.85 (s, 3H); ^{13}C NMR (75.4 MHz, DMSO-d_6) δ 158.6, 160.4, 159.1, 129.7, 121.8, 115.3, 115.0, 55.8, 55.6; HRMS (EI) calcd for $C_9H_8N_2O_3$ 192.0529 [M-N_2]$^+$, found 192.0531.

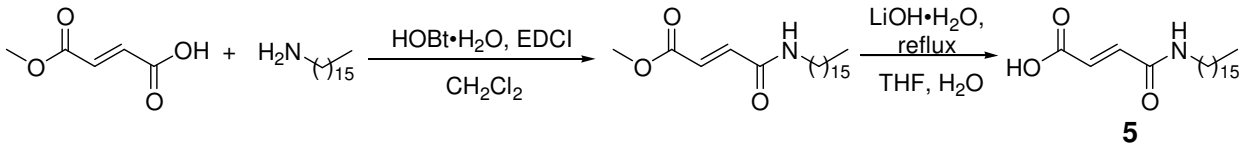


2-(9*H*-Fluoren-9-ylmethoxycarbonylamino)-6-{[2-(4-methoxy-phenyl)-2*H*-tetrazole-5-carbonyl]-amino}-hexanoic acid: To a solution of 2-(4-methoxy-phenyl)-2*H*-tetrazole-5-carboxylic acid (0.500 g, 2.1 mmol) in 50 mL anhydrous CH_2Cl_2 was added DMF (5 mol%) and 5 equiv of oxalyl chloride dropwise. The mixture was stirred for 2 hr before the solvent and excess reagents were removed under reduced pressure. The residue was further dried in vacuum for 5 hr to yield the tetrazole acid chloride. To a solution of Fmoc-Lys-OH • HCl (0.85 g, 2.1 mmol) and DIEA (4.2 mmol) in 600 mL dioxane cooled in an ice-water bath was added simultaneously tetrazole acid chloride (dissolved in 20 mL anhydrous THF) and DIEA (4.2 mmol, dissolved in 20 mL anhydrous THF) via a syringe pump at a rate of 50 μL/min. Following addition the reaction was allowed to stir overnight. The crude reaction mixture was concentrated and then re-dissolved in 100 mL CH_2Cl_2. The solution was washed 3 times with 10% $KHSO_4$, and the organic layer was separated before evaporation under the reduced pressure. The residue was applied to a silica gel column using 1:20 MeOH/CH_2Cl_2 as an eluent to afford the titled compound as a slight yellow foam (0.781 g, 67%): ^{1}H-NMR (500 MHz, DMSO-d_6) δ 9.15 (t, J = 3.4 Hz, 1H), 8.03 (d, J = 9.0 Hz, 2H), 7.88 (d, J = 7.5 Hz, 2H), 7.71 (d, J = 7.0 Hz, 1H), 7.65 (d, J = 8.0 Hz, 1H), 7.39 (t, J = 7.5 Hz, 2H), 7.30 (t, J = 8.0 Hz, 2H), 7.21 (d, J = 9.5 Hz, 2H), 4.25 (m, 3H), 4.19 (m, 1H), 3.87 (s, 3H), 1.72-1.55 (br, m, 5H), 1.38 (m, 3H); ^{13}C-NMR (75.4 MHz, DMSO-d_6) δ 173.9, 160.7, 159.9, 156.1, 143.8, 140.7, 129.3, 127.6, 127.0, 125.2, 122.0, 120.1, 115.1,68.1, 55.7, 53.6, 46.6, 30.4, 28.4, 23.1; HRMS (ESI) calcd for $C_{30}H_{31}N_6O_6$ 571.2305 [M+H]$^+$, found 571.2303.

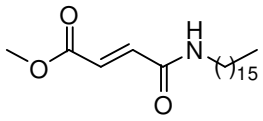
Synthesis of Tetrazole Tail 1 and 2: The tetrazole tails were synthesized manually in a polypropylene reaction vessel by following the standard Fmoc-based solid phase peptide synthesis protocol. Fmoc-Gly-Wang resin (Advanced ChemTech, 0.97 mmol/g) was used. For coupling of Fmoc-Lys(Tet)-OH, two equiv of amino acids, two equiv of HBTU/HOBt, and eight equiv of DIEA in DMF were used, and the

reactions were allowed to proceed for 1 hr. For coupling of natural amino acids, three equiv of amino acids, three equiv of HBTU/HOBt, and six equiv of DIEA in DMF were used, and the reactions were allowed to proceed for 45 min. The Fmoc deprotection was accomplished by treating the resin-bound peptides with 20% piperidine/DMF (2×, 10 min each). The peptides were cleaved from the Wang resin by treating the resin with 1 mL cleavage cocktail (94% TFA, 2.5% water, 2.5% ethanedithiol, 1% triisopropylsilane) for 1 hr. The cleavage solutions were filtered into cold ether and the precipitated crude peptides were found to be greater than 90% pure by analytical HPLC and LC-MS. **Tet 1**: ESI-MS calcd for $C_{20}H_{29}N_8O_6S$ 509.2 $[M+H]^+$, found 509.1. **Tet 2**: ESI-MS calcd for $C_{35}H_{47}N_{14}O_9S$ 839.3 $[M+H]^+$, found 839.2.

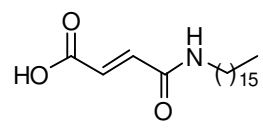
Synthesis of *N*-palmityl fumaric acid 5:



5



(*E*)-Methyl 4-(hexadecylamino)-4-oxobut-2-enoate: The solution of the mono-methyl fumarate (500 mg, 3.80 mmol), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDCI, 745.2 mg, 4.80 mmol) and mono-hydrate hydroxylbenzo-triazole ($HOBt{\cdot}H_2O$, 582.1 mg, 3.80 mmol) in 20 mL CH_2Cl_2 was stirred for 20 min at room temperature. Then a solution of hexadecan-1-amine (965.8 mg, 3.80 mmol) in 20 mL CH_2Cl_2 was added dropwise. After 12 h, the solution was diluted with CH_2Cl_2 and filtrated; 20 mL water was added and washed. The mixture was washed with saturated $NaHCO_3$, brine, dried over Na_2SO_4. The residue was purified *via* silica gel column to obtain the title compound as a glossy white solid (820.0 mg, 61.0% yield): ^{1}H-NMR (500 MHz, $CDCl_3$) δ 6.90 (d, J = 15.5 Hz, 1H), 6.83 (d, J = 15.0 Hz, 1H), 5.85 (br., 1H), 3.80 (s, 3H), 3.35 (q, J = 7.0 Hz, 2H), 1.56-1.52 (m, 2H), 1.30-1.25 (m, 26H), 0.88 (t, J = 7.0 Hz, 3H); ESI-MS calcd for $C_{21}H_{39}NNaO_3$ $[M+Na]^+$ 376.3, found 376.2.

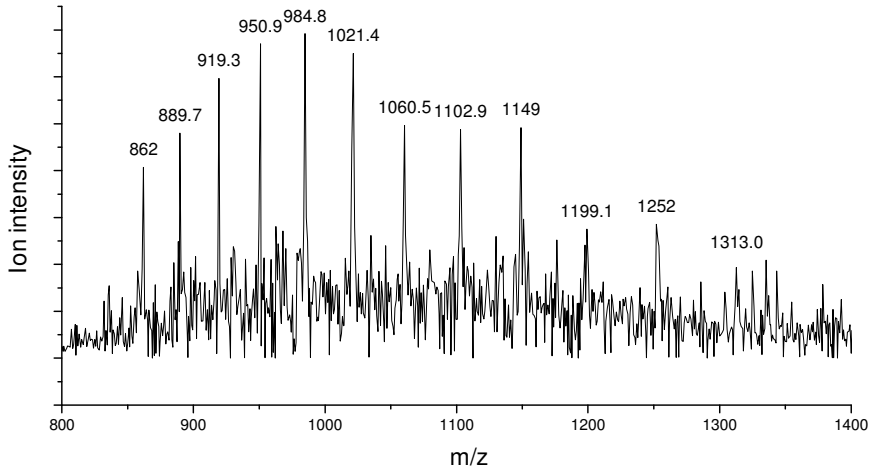


(*E*)-4-(Hexadecylamino)-4-oxobut-2-enoic acid (5): To a solution of (*E*)-methyl 4-(hexadecylamino)-4-oxobut-2-enoate (575.5 mg, 1,63 mmol) in THF-H_2O (10 mL THF, 4 mL H_2O) was added monohydrated lithium hydroxide (136.8 mg, 3.25 mmol). The mixture was refluxed until the starting material disappeared as determined by TLC. The solvent was removed under vacuum. The residue was dissolved in water and washed with 50 mL EtOAc. The solution was acidified with 1.0 M HCl and the precipitate was filtrated and dried in vacuo. The crude product was recrystallized in THF to give the desired product as a white solid (520 mg, 94% yield): ^{1}H-NMR (500 MHz, CD_3OD-d_4) δ 8.56 (br, 1 H), 6.98 (d, J = 15.5 Hz, 1H), 6.70 (d, J = 15.5 Hz, 1H), 3.28

(q, J = 7.1 Hz, 2H), 1.60-1.55 (m, 2H), 1.36-1.30 (m, 26H), 0.92 (t, J = 7.1 Hz, 3H); ESI-MS calcd for $C_{20}H_{38}NO_3$ $[M+H]^+$ 340.3, found 340.4.

Construction of pTXB1-EGFP plasmid: The EGFP fragment carrying *Nde* I and *Sap* I restriction site at the 5'- and 3'-end, respectively, was amplified through a PCR reaction using pEGFP-C1 as the template and the following two primers: 5'-GGTGGTCATATGGTGAGCAAGGGCGAGG-3' and 5'-GGTGGTTGCTCTTCCGCACTTGTACAGCTCGTCC-3' (restriction sites underlined). After gel purification, the EGFP fragment was digested with *Nde* I and *Sap* I in the NEBuffer 4 at 37°C for 2 hr. The digested EGFP fragment was purified by 1% agarose gel and incubated with the pTXB1 vector pre-digested with *Nde* I/*Sap* I and T4 DNA ligase at 14°C overnight. Afterwards, 1 µL of the ligation mixture was used to transform MaxEfficiency DH5α competent cells (Invitrogen), and the transformants were selected on an LB agar plate containing 50 µg/mL ampicillin.

Preparation of EGFP-Tet 3 and 4: pTXB1-EGFP plasmid was transformed into the Rosetta competent cells (Novagen) using the heat-shock method and plated onto LB agar plate containing 50 µg/mL ampicillin. The single colony picked from the plate was allowed to grow in 20 mL LB medium supplemented with 50 µg/mL ampicillin at 37 ºC. The overnight culture was diluted into 1000 mL fresh LB medium containing 50 µg/mL ampicillin and allowed to grow at 30 ºC until OD600 reached 0.6. Induction of EGFP was initiated by adding 0.3 mM IPTG and the culture was allowed to grow overnight. The cells were harvested and the EGFP-intein-CBD fusion protein in the supernatant was captured onto a freshly prepared affinity column loaded with 1.2 mL chitin beads followed by 5× washing with 10 mL each of wash buffer (20 mM Tris, 500 mM NaCl, 1 mM EDTA, pH 7.5). The beads were treated with 2 mL of cleavage buffer (wash buffer supplemented with 3 mM Tet **1** or **2** and 50 mM MESA) for 48 hr at 4 ºC. The filtrate was applied to Amicon Ultra-4 10K device (Millipore) to remove excess amount of small molecules and salts, then purified by FPLC (Mono Q 5/50, GE Healthcare). The fractions were examined by SDS-PAGE, and the pure fractions were pooled to give EGFP-Tet **3** and EGFP-Tet **4**, respectively. ESI-MS calcd for EGFP-Tet **3**·MESA 27536.9 Da, found 27547.7 ± 8.7 Da.



z	m/z	m
32	862.0	27553.3
31	889.7	27550.3
30	919.3	27547.8
29	950.9	27546.5
28	984.8	27545.6
27	1021.4	27551.6
26	1060.5	27547.0
25	1102.9	27547.5
24	1149.0	27553.0
23	1199.1	27555.4
22	1252.0	27522.0
21	1313.0	27552.0
av		27547.7
std		8.7

Lipidation of EGFP-Tet via Photoinduced Cycloaddition Reaction: To 20 μL samples of either 7 μM EGFP-Tet **3** or EGFP-Tet **4** in DPBS buffer in a 96-well microtiter plate were added 1 μL of lipid dipolarophile **5** (5 mM in DMSO; final concentration = 250 μM). After photoirradiation with a hand-held 302 nm UV lamp for 5 min, 5 μL of 6× SDS sample buffers were added and the mixtures were boiled at 95 °C for 5 min. The samples were loaded onto a NuPAGE 12% Bis-Tris gel and resolved by SDS-PAGE. The EGFP-Pyr cycloadducts in the gel were visualized by illuminating the gel with a hand-held 365 nm UV lamp, and recorded with a digital camera. Afterwards, the gel was stained with Coomassie Blue to confirm the equal protein loadings.

Partitioning of EGFP-Tet 3 and 4 Between the Organic Phase and the Aqueous Phase After the Photoinduced Cycloaddition Reaction: To a calculated 20 μM of EGFP-Tet **3** or **4** in 240 μL DPBS buffer, pH 7.5, in a quartz test tube was added 10 μL lipid dipolarophile **5** (10 mM in DMSO). Under vigorous stirring the mixture was irradiated with a handheld 302-nm UV lamp for 2 min. Afterwards, 10 μL of either reaction product (EGFP-Pyr) or starting materials (EGFP-Tet) was added into a mixed solvent containing 400 μL DPBS and 750 μL dichloromethane in a 1.5-mL eppendorf tube. The mixture was vigorously shaken before the solution was allowed to settle into two layers. The localization of EGFP was monitored with fluorescence by irradiating the samples with a handheld 365-nm UV lamp from the top.

Microinjection of EGFP-Tet 4: Microinjection samples were prepared by mixing EGFP-Tet **4** (25 μM) with Dextran-TMR (2 μM) (Dextran-tetramethylrhodamine, MW ~70 KDa, Invitrogen) in a micro-injection buffer (2 mM HEPES, 150 mM KCl, 0.2 mM EGTA, pH 7.0). Dextran-TMR was used as a red fluorescent marker that is excluded from the nucleus when injected in the cytoplasm. Cells were imaged for up to 4 h after microinjection. The morphology of the microinjected cells did not change dramatically

during image acquisition; the intracellular fluorescence intensities measured at different time points were found to be constant, indicating that the injected protein is not toxic to cells and not degraded during the experimental time frame. 200 μL of lipid dipolarophile **5** (1 mM in DPBS) was added to 2 mL low fluorescent culture medium (without phenol red) and cultured for 15 min before photoirradiation with a hand-held 302 nm UV lamp for 1 min. Images were acquired 1 min after photoirradiation.

Live-Cell Imaging: HeLa cells were cultured on a 35-mm plate (P35G-1.5-14-C, MatTek Corp., Ashland, MA) at 2.5×10^5 cells in a total volume of 2 mL of Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS in a humidified incubator containing 5% CO2. For live-cell imaging, cells were washed and incubated with low fluorescent medium (1× Earle's Balanced Salt Solution with 1× MEM Non-Essential Amino Acids solution, 1× MEM Amino Acids solution, and 1× L-glutamine) supplemented with 10% FBS. A confocal microscope (Zeiss LSM 510 Meta NLO Confocal Microscope attached Zeiss Axiovert 200M) equipped with a controlled chamber maintaining 37°C, 5% CO2 and humidity was used for image acquisition. Images were collected using a Hamamatsu ORCA ER digital CCD camera mounted on the microscope equipped with a 63×/1.4 Oil DIC objective, and filter sets with excitation wavelengths of 488 and 561nm and emission wavelengths of 500-550 nm and 575-615 nm. The photoinduced cycloaddition reaction with the microinjected proteins was performed with a hand-held 302 nm UV lamp (UVP: UVM-57, 0.16 Amps). A control experiment showing intracellular localization of EGFP-Tet**4** in the presence of **5** before the photoirradiation is as follows: