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Supporting Information

New Water-soluble Alkynylating Agent for Cell Surface Protein: Sulfosuccinimidyl 4-Pentynoate

Choon Woo Lim, Hoa Thi Le, Ji Hye Han, Dong-Hwan Kim, Jae Gyu Jang, and Tae Woo Kim*

Graduate School of East-West Medical Science, Kyung Hee University, Gyeonggi-do 449-701, Korea
**E-mail: tw1275@ khu.ac.kr*
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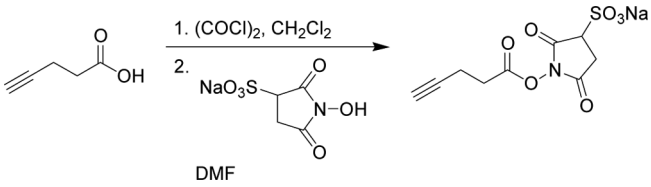
Synthesis and Characterization of Sulfo-NHS-E

General: All reagents were purchased from Aldrich and used without purifications. ^{1}H-, ^{13}C-NMR spectra were measured on JNM-AL300 (JEOL) spectrometer. Chemical shifts were reported as δ scale in ppm (parts per million) relative to H_2O (δ 4.65), and *J*-values are in Hz. High resolution mass spectrum was measured on JMS-600W (JEOL) mass spectrometer. THPTA (tris(3-hydroxypropyl-triazolylmethyl)amine) was prepared by the reported Finn's procedure.[1]



Scheme S1. The synthesis of Sulfo-NHS-E.

Sulfo-NHS-E: 4-pentynoic acid (680 mg, 6.9 mmol) was dissolved in anhydrous methylene chloride (5 mL). Oxalyl chloride (2.3 mL, 4.6 mmol, 2 M solution in CH_2Cl_2) was injected to the solution and the reaction mixture was refluxed for 1 h. The solvent was carefully evaporated in ice bath (boiling point of the acid chloride of 4-pentynoic acid, 144.9 °C at 760 Torr; boiling point of oxalic chloride, 63-64 °C at 760 Torr). The solution of sulfo *N*-hydroxysuccinimide (250 mg, 1.2 mmol) in anhydrous DMF (10 mL) was injected to the residue. The reaction mixture was stirred for 24 h at room temperature.

The solvent was evaporated under rotary evaporator and on high vacuum. The residue was suspended in minimum amount of anhydrous methanol and triturated with diethyl ether. The precipitate was collected by centrifugation (2,000 rpm, 5 min). The additional trituration (3 times in diethyl ether) gave the desire product (white solid, 250 mg, 0.84 mmol, yield 70%).

^{1}H NMR (D_2O, 300 MHz) δ 4.30 (br d, $J_{H1\text{-}H2}$ = 7.3 Hz, 1H, H_1), 3.20 (dd, $^2J_{H2\text{-}H3}$ = 19, $J_{H2\text{-}H1}$ = 8.8 Hz, 1H, H_2), 3.01 (dd, $^2J_{H3\text{-}H2}$ = 19, $J_{H3\text{-}H1}$ = 3.1 Hz, 1H, H_3), 2.79 (t, $J_{H4\text{-}H5}$ = 6.8 Hz, 2H, H_4), 2.44 (td, $J_{H5\text{-}H4}$ = 6.8, $^4J_{H5\text{-}H6}$ = 2.6 Hz, 2H, H_5), 2.20 (t, $J_{H6\text{-}H5}$ = 2.7 Hz, 1H, H_6); ^{13}C NMR (DMSO-d_6, 75 MHz): 168.5, 167.4, 165.1, 81.9, 72.1, 56.2, 30.8, 29.6, 13.4; HRMS (FAB+, glycerol): calcd mass 296.9919 for [$C_9H_8NNaO_7S$], found 297.9997 for $[M+H]^+$.

The ^{1}H NMR spectrum of sulfo *N*-hydroxysuccinimide shows three peaks with 1:1:1 integration ratio at δ 4.18 (dd, *J* = 8.8, 3.1 Hz), 3.06 (dd, *J* = 18.7, 8.8 Hz), 2.87 (dd, *J* = 18.7, 3.1 Hz) (Fig. S1). The largest coupling constant (*J* = 18.7 Hz) results from a geminal spin-spin coupling ($^2J_{ab}$). The middle one (*J* = 8.8 Hz) comes from a vicinal spin-spin coupling constant ($^3J_{bc}$) between two proton H_b/H_c having ~0° dihedral angle. The smallest one (*J* = 3.1 Hz) does from a vicinal spin-spin coupling constant ($^3J_{ac}$) between two proton, H_a/H_c having ~120° dihedral angle. In the same manner, we can assign the exact proton position of Sulfo-NHS-E from the spin-spin coupling constant analysis. In addition the terminal alkyne proton and the triple bond adjacent CH_2 proton show the characteristic propargyl spin-spin coupling constant (^4J = 2-4 Hz).

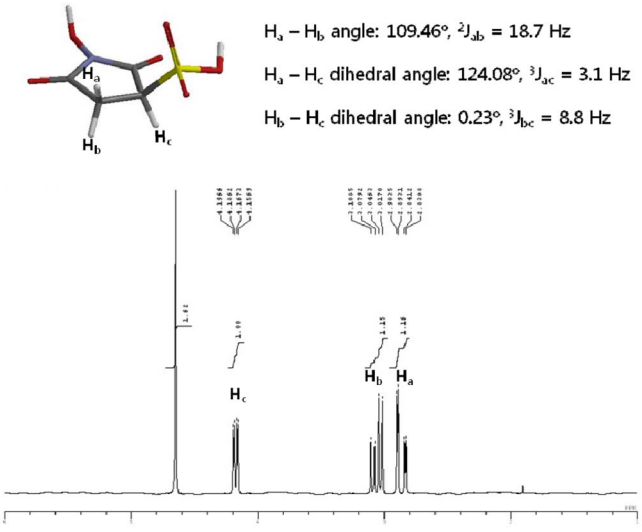


Figure S1. The energy minimized structure (Spartan '08, Wavefunction Inc.) and spin-spin coupling analysis of sulfo *N*-hydroxysuccinimide.

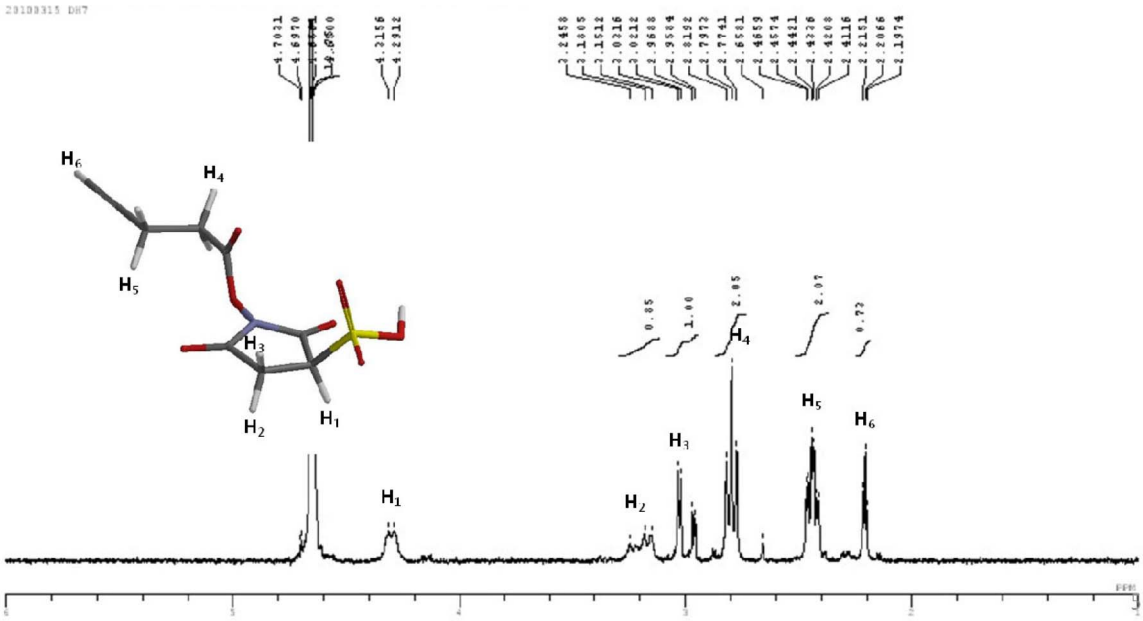


Figure S2. ^{1}H NMR spectrum (D_2O, 300 MHz) of Sulfo-NHS-E.

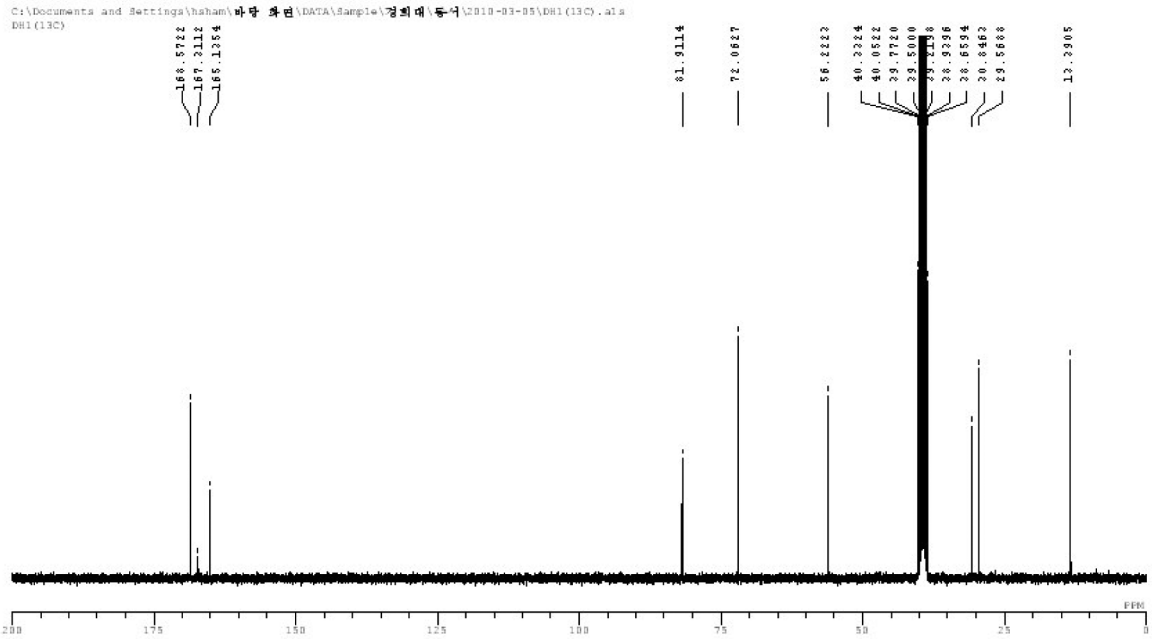
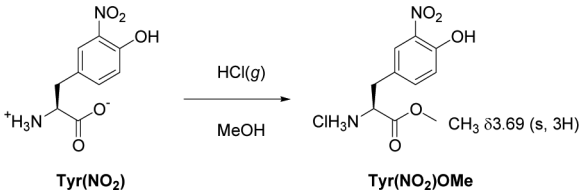


Figure S3. ^{13}C NMR spectrum (DMSO-d_6, 75 MHz) of Sulfo-NHS-E.

Amidation Kinetics: Amide Bond formation between Sulfo-NHS-E (an activated ester) and Tyr(NO_2)-OMe (an amine)

Tyr(NO_2)-OMe Preparation: To monitor the reaction kinetics with HPLC, the reaction partner of Sulfo-NHS-E have two requirements: 1) detectable UV/visible absorption, 2) moderate hydrophobicity for reverse phase column. 3-Nitro-L-tyrosine methyl ester was proposed and 3-Nitro-L-tyrosine methyl ester hydrochloride (Tyr(NO_2)-OMe) was prepared from 3-Nitro-L-tyrosine (Tyr(NO_2)) by acid catalyzed esterification following the literature procedure.[2]

UV/visible Absorption of Tyr(NO_2)-OMe: Because of 0.1% TFA in water, basically quenched sample became acidified during HPLC analysis. As a result, the equilibrium of **A**, **B**, and **C** made the chromatogram complicate (Fig.



Scheme S2. The synthesis of 3-Nitro-L-tyrosine methyl ester hydrochloride.

S4). Thus the reaction mixture was quenched with acid and analyzed by HPLC. The pK_a of phenolic proton is 7.1.[3]

Time Dependent Reaction Monitoring: For the reaction monitoring, 1 mL of Tyr(NO_2)-OMe (1 mM) in 2xPBS (pH 7.4) was mixed with Sulfo-NHS-E (1.4 mg in 1 mL water), and shaken in an Eppendorf thermomixer (1,200 rpm) at 25 °C. After time intervals, 100 μL of reaction mixture was sampled and quenched with 20 μLof 1M HCl. The samples were analyzed with RP HPLC (column: 4.6 mm ID × 150 mm Extend-C18 column (5 μm pore size; Hewlett Packard,

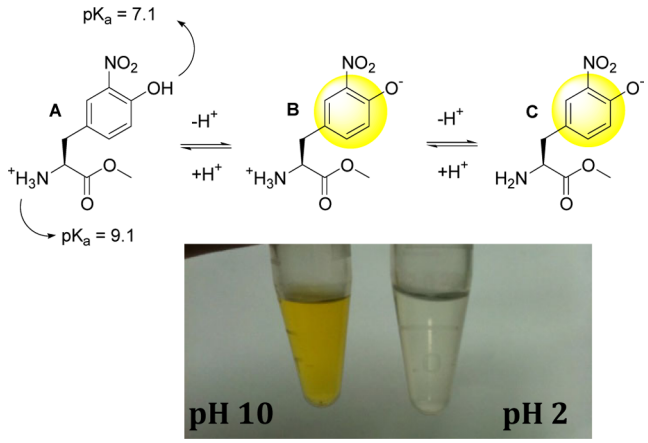


Figure S4. pH dependent protonation/deprotonation of Tyr(NO_2)-OMe and pH dependent color change.

CA); detector: UV-DAD; eluent: CH_3CN/water (0.1% TFA); condition: linear gradient 10%- 60%, 20 min).

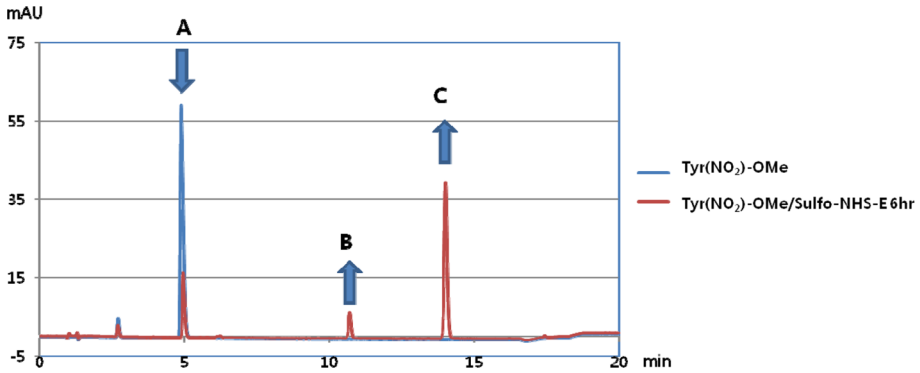
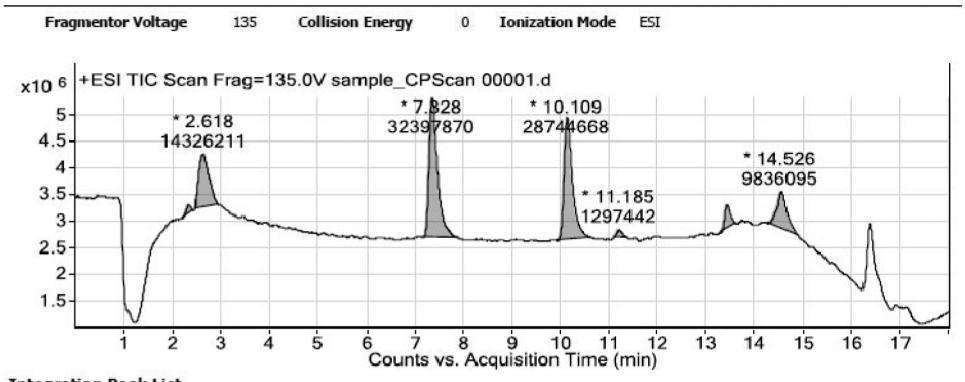


Figure S5. Overlapped chromatograms of Tyr(NO_2)-OMe standard and of the sample after 6 h reaction. A: Tyr(NO_2)-OMe; B: E-Tyr(NO_2); C: E-Tyr(NO_2)-OMe (SEE Fig. S7).



Integration Peak List

Peak	Start	RT	End	Height	Area	Area %
1	2.179	2.308	2.411	179012	1282134	3.96
2	2.471	2.618	2.945	1001853	14326211	44.22
3	7.147	7.328	7.879	2614126	32397870	100
4	9.894	10.109	10.643	2305405	28744668	88.72
5	11.074	11.185	11.375	148840	1297442	4
6	13.278	13.416	13.614	449598	3945699	12.18
7	14.26	14.526	14.862	691542	9836095	30.36

Figure S6. Total Ion Current (TIC) Chromatogram of the reaction mixture in LC/MS (Ionization Mode: ESI-positive).

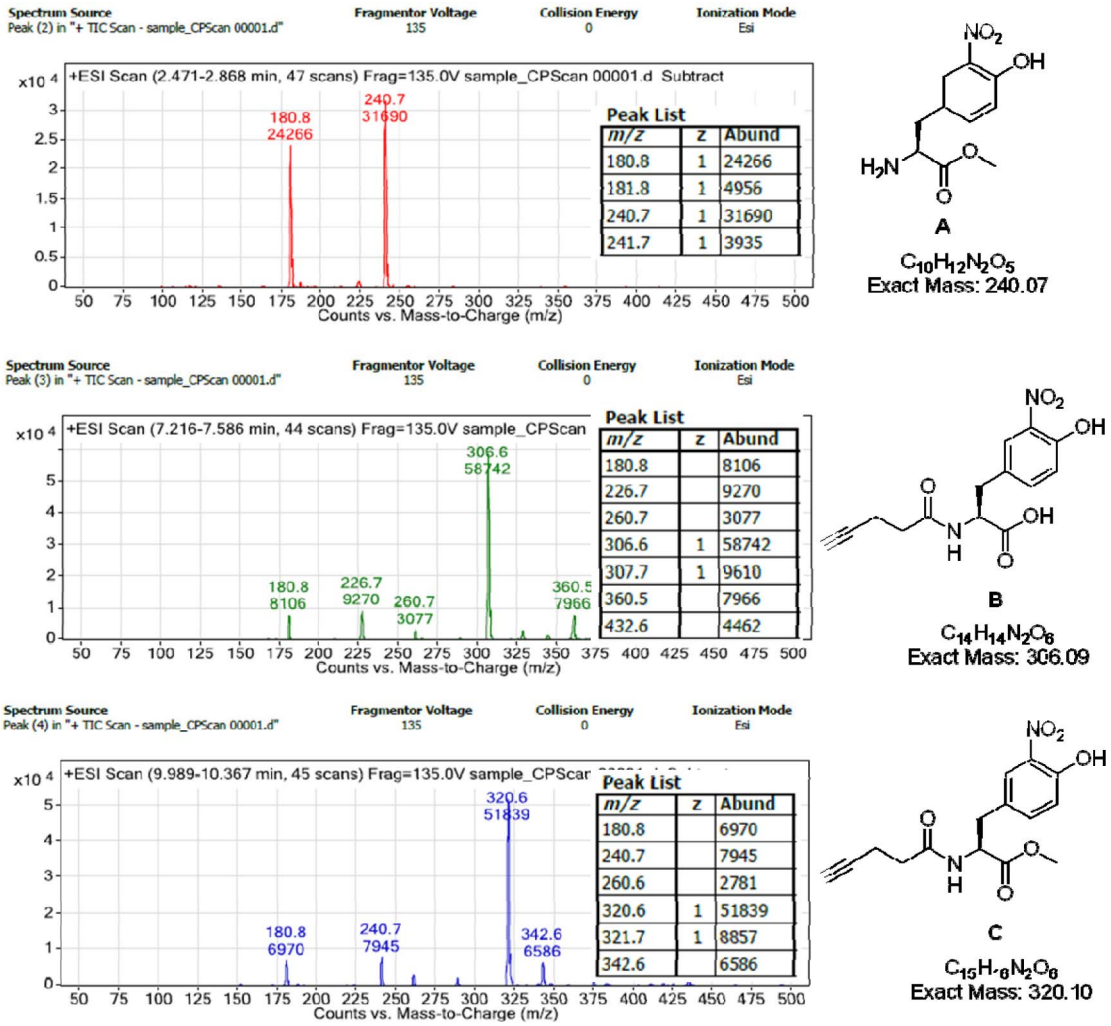


Figure S7. Peak assignment in LC/MS: Peak 2 (RT = 2.618) = Tyr(NO_2)-OMe, Peak 3 (RT = 7.328) = E-Tyr(NO_2), Peak 4 (RT = 10.109) = E-Tyr(NO_2)-OMe: RT = retention time.

Peak B was assumed to a hydrolyzed form of E-Tyr(NO_2)-OMe in acid quenching condition and confirmed to E-Tyr(NO_2) by HPLC-MS analysis (Fig. S6 and S7). The reaction percentage in Fig. 2 was calculated following equations:

Relative peak area % of Tyr(NO_2)-OMe = (peak area of A / Σ peak areas of A, B, C) × 100

Relative peak area % of ethynylated Tyr(NO_2)) = ((peak area of B + peak area of C)/ Σ peak area of A, B, C) × 100.

Fluorescence labeling of HepG2 cell surface protein and it's confocal fluorescence imaging

Cell culture: HepG2 cells, a human hepatoma cell line, were cultured in DMEM supplemented with 10% FPB and 1% penicillin-streptomycin at 37 °C in 5% CO_2. Cells were seeded on 24-well plates and stabilized for overnight.

Cell surface protein ethynylation: HepG2 cells in 24-well plate (1×10^5 cells/well) was cultivated and stabilized for overnight. The cells were washed twisce with ice-cold 1xPBS (1 mL) per well. Sulfo-NHS-E (2 mg) was dissolved in ice-cold 1xPBS (10 mL) and the freshly prepared solution (1 mL) was treated to each well. The plate was gently agitated for 30 min 4 °C on an orbital shaker. The cells were washed three times with 1xPBS.

Fluorescence labeling with Click reaction: 1 μL of Alexa 488 azide stock solution (0.5 mg/70 μL DMSO, Life Technologies Cat. No. A10266) was added to click cocktail ($CuSO_4$/THPTA (1:5 ratio, 1 mM), sodium ascorbate (5 mM, freshly prepared before use), aminoguanidine hydrochloride (5 mM) in phosphate buffer (0.1 M, pH 7.4)). The solution was treated onto the cells and then incubated for 10 min. The cells were washed three times with 1xPBS and imaged using confocal microscopy.

Confocal Microscopy Imaging: The fluorescence images were taken with a 200 × 4 objective using a confocal laser scanning microscope (Zeiss LSM 510, Zeiss, Oberko, Germany) which was equipped with a 488 nm Argon laser and a 505-nm long pass filter.

Protein Ethynylation and SDS PAGE

Protein mixture Ethynylation: 10 μL of proteins standard (Bio-rad cat. no. 161-0317) was added to 500 μL of 1xPBS to the final protein concentration, 0.36 μg/μL. 1 μL of freshly prepared Sulfo-NHS-E stock (1 mg/mL) was added to the protein mixture and incubated at 25 °C for 30 min. The amidation was quenched by the addition of excess glycine. To remove the unreacted Sulfo-NHS-E and the excess glycine, the buffer was exchanged with an Amicon Ultra-0.5 centrifugal filter (Millipore UFC5010, Ultracel-10 membrane, 10 kDa MWCO: 14,000 g, 4 °C, 15 min, 3 times). The residue was diluted to 100 μL with 1xPBS buffer.

Cell lysate preparation: HepG2/C6 cells were cultured in DMEM supplemented with 10% FPB and 1% penicillin-streptomycin at 37 °C in 5% CO_2. The cells were seeded on 24-well plates and stabilized for overnight. The gently scraped cells were transferred into a 50ml conical tube. The Petri dish was rinsed with 1xPBS (combined total volume, 10 mL) and the rinsed solution was added to the 50 ml conical tube. The cells were pelleted by centrifugation (500 × g, 2 min) and the supernatant was discarded. The cells were lysated using 2% Triton x-100 (containing 1% PIC) and sonication for 2 min (5 sec pulse). To remove cell debris, the lysate was centrifuged at 10,000 × g for 2 min and the supernatant was transferred to new tube. The amount of proteins was quantified by BCA (bicinchoninic acid) assay.

Click reaction of the ethynylated protein standard & SDS PAGE: The above ethynylated protein standard (10 μL), Alex488-N_3 (Life technologies, cat. no. A10266; 5 μL, stock: 0.7 μg/μL in DMSO), and the cell lysate (15 μL, stock: 1,160 μg/mL of C6 or stock: 300 μg/mL of HepG2) were diluted in 10xPBS and water (final reaction volume = 50 μL). After mixing, the premixed $CuSO_4$:THPTA (5 μL, stock: 10 mM, 1:5 ratio) was added. Finally, freshly prepared sodium ascorbate (5 μL, stock: 50 mM) was added. The reaction mixture was shaken at 37 °C for 40 min in an Eppendorf thermomixer. After 1 hour, an aliquot (10 μL) of the reaction mixture was mixed with gel loading buffer (10 μL; Tris-HCl (pH 6.8) 60 mM, glycerol 10%, SDS 2%, bromophenol 0.5%, β-merccaptoethanol 5%) and the mixture was heated at 95 °C for 5 min. The denatured 20 μL sample was loaded on a 10% SDS-polyacrylamide gel (10% acrylamide mix (acrylamide:*N,N′*-methylenebisacrylamide = 30:1), Tris-HCl (pH 8.8) 0.38 M, SDS 0.1%) and ran. The gel was then treated 3 times for 15 min with a fixing solution (2% phosphoric acid in 30% ethanol) and 3 times for 10 min with a rinsing solution (2% phosphoric acid in 5% ethanol). The fluorescence was imaged with a Molecular Dynamics Typhoon 8600. The recovered gel was treated for 30 min with an equilibrium buffer (Tris-HCl (pH 8.8) 50 mM, urea 6 M, glycerol 30%, SDS 2%) and stained overnight in a staining solution (0.02% CBB G250, 5% $Al_2(SO_4)_3$, 5% ethanol, and 2% phosphoric acid). The stained gel was washed with water and scanned.

References

1. Hong, V.; Presolski, S. I.; Ma, C.; Finn, M. G. *Angew. Chem. Int. Ed*. **2009**, *48*, 9879.
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