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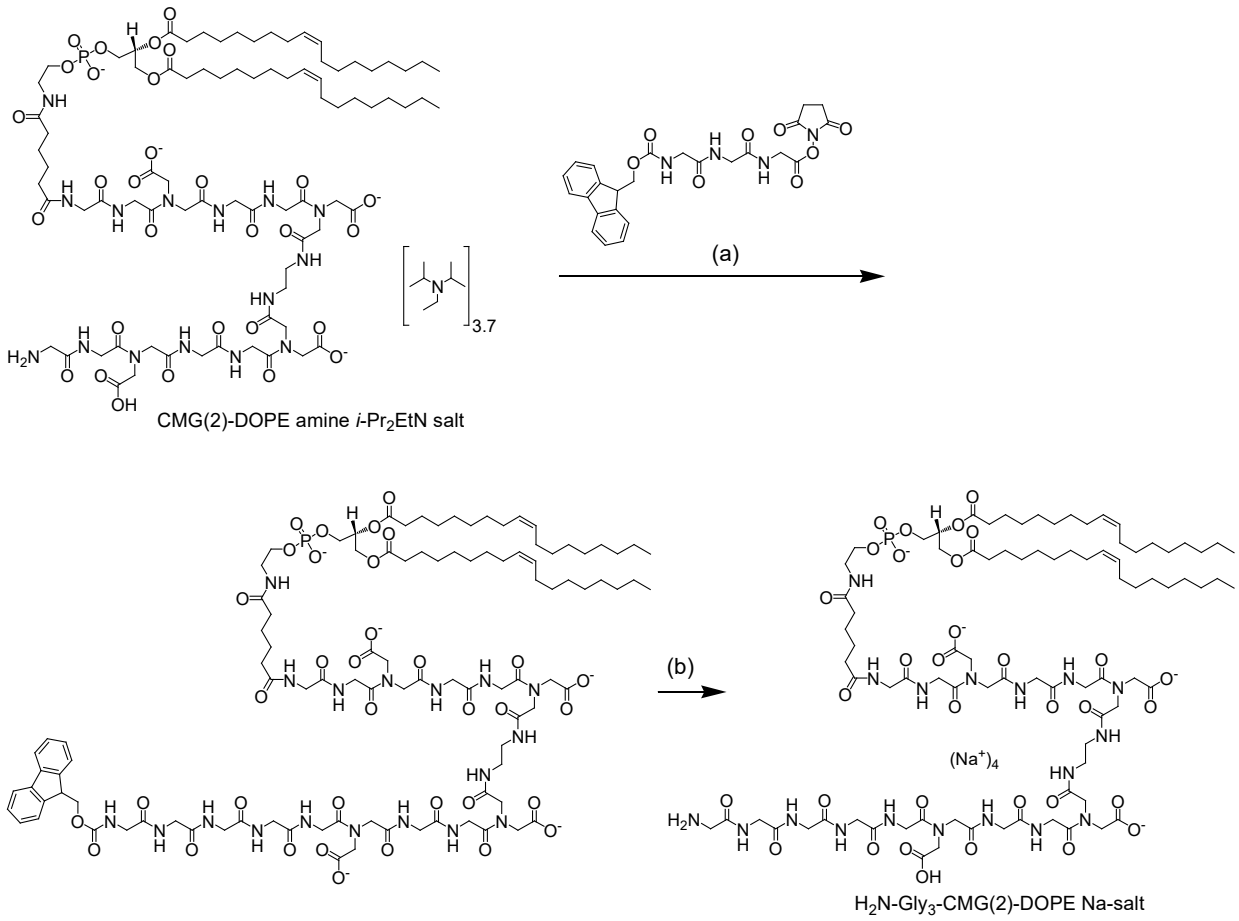
Site-specific sortase-catalyzed lipidation of proteins

Tatiana D. Melikhova, Nicolai V. Bovin, Natalia S. Shoshina, Tatiana V. Bobik, Leonid A. Gavrilov, Marina A. Sablina, Eugenia M. Rapoport, Vasiliy N. Stepanenko and Alexander B. Tuzikov

1. Materials and Instrumentation

All chemicals used were obtained from commercial sources and used without further purification: 1,4-dithiothreitol (DTT), tris(hydroxymethyl)aminomethane, NaCl, sodium dodecyl sulfate (SDS) from Fluka AG (Switzerland); *N,N*-methylenebisacrylamide, acrylamide, ammonium persulfate, tetramethylethylene diamine (TEMED) from Bio-Rad (USA), triglycine and other chemicals for synthesis were from Sigma if not specified. Reagents for preparation of buffers were from Panreac (Spain), unless otherwise stated. Fmoc-Gly_3, *N,N*-diisopropylethylamine and Sephadex LH-20 were from Sigma-Aldrich (USA), *N,N′*-dicyclohexylcarbodiimide and *N*-hydroxy-succinimide were from Merck (Germany), CMG(2)-DOPE from KODE Biotech (New Zealand). TLC was performed using precoated Kieselgel 60 F_{254} aluminum sheets (Merck, Darmstadt, Germany); detection under UV light, visualization by charring with 7% phosphoric acid (200–250 °C) or treatment with 2% solution of ninhydrin in 2-propanol.

The following devices were used: Cell disintegrator Branson Sonifier 450 (Branson Ultrasonics Corporation, USA), centrifuge BioFuge (Heraeus Sepatech, Germany), chromatographic systems Biologic LP (BioRad, USA) and Waters 2489/1525 (Waters Corporation, USA), electrophoresis equipment from BioRad, USA, shaker G24 (New Braunswick Scientific, USA), FACScan (Becton Dickinson, USA). NMR spectra were recorded on Bruker Avance 700 or Bruker Avance 800 (Bruker BioSpin MRI GmbH) spectrometers. High resolution mass spectra (HRESIMS) were measured on a Bruker microTOF II instrument using electrospray ionization (ESI).



Scheme S1. Synthesis of H_2N-Gly_3-CMG(2)-DOPE. (*a*) Fmoc-Gly_3 NHS ester (1.1 eq.) in DMF/DMSO, Pr^i_2NEt (1.3 eq.), 1 h / r.t.; (*b*) piperidine, 40 min / r.t., isolation on Sephadex LH-20 in 2-propanol/water 1:2.

2. Synthesis of H_2N-Gly_3-CMG(2)-DOPE

To a stirred solution of CMG(2)-DOPE $(Pr^i_2EtN)_{3.7}$ salt (120 mg, 0.052 mmol) in DMSO (0.3 mL) a solution of Fmoc-Gly_3 NHS ester in DMF (0.057 mmol in 0.6 mL, prepared from Fmoc-Gly_3, *N*-hydroxysuccinimide and *N,N′*-dicyclohexylcarbodiimide 1:1:1 by mole without isolation, *N,N′*-dicyclohexylurea was filtered off) and Pr^i_2EtN (0.069 mmol, 12 μL) were added (Scheme S1). After 1 h at room temperature, piperidine (60 μL) was added, and the solution was kept for 40 min at room temperature. AcOH (52 μL) and 2.5 M NaOAc (0.4 mL) were added and the mixture was diluted with minimal volume of water to clear solution. The solution was applied on Sephadex LH-20 column (140 mL, the column was previously washed with 2-propanol/water 1:2 after application of 0.5 ml 2.5 M NaOAc). Selected fractions were evaporated, the residue was freeze-dried from water to give 93 mg (86% yield) of the product as a white solid.

TLC: R_f 0.58 ($CHCl_3$/MeOH/water, 1:3:1). ^{1}H NMR (700 MHz, DMSO-d_6/D_2O 3:1, 303 K) (Figure S1) δ: 5.327 (m, 4H, 2 CH=CH), 5.106 (m, 1H, 2-CH of glycerol), 4.324 (dd, 1H, CH-1 of glycerol, *J* 12.2 Hz, *J* 2.6 Hz), 4.124 (s, HOD), 4.093 (dd, 1H, CH'-1 of glycerol, *J* 12.2 Hz, *J* 4.9 Hz), 4.026−3.683 (m, total 38H, 4 $CH_2C(O)O$, 13 NCH_2CO, 3-CH_2 of glycerol, $POCH_2$), 3.636 (br. s, 2H, $COCH_2NH_2$), 3.229 (t, 2H, NCH_2CH_2O, *J* 5.8 Hz), 3.169-3.113 (m, 4H, NCH_2CH_2N), 2.563 (p, D_5H-DMSO, *J* 1.84 Hz), 2.316-2.218 (m, 4H, 2 $CH_2C(O)$), 2.185 (m, 2H, $CH_2C(O)$), 2.115 (m, 2H, $CH_2C(O)$), 1.972 (m, 8H, 2 $CH_2C=CCH_2$), 1.501 (m, 8H, 4 $CH_2CH_2(CO)$), 1.297-1.239 (m, 40H, 20 CH_2 of Ole), 0.846 (t, 6H, 2 CH_3, *J* 6.9 Hz) ppm. ^{13}C NMR (D_2O/CD_3OD 2:1, 303K, 201 MHz) (Figure S2): δ 176.7−170.7 (C=O), 129.69 and 129.58 (2 CH=CH), 70.7 and 70.6 and 64.0 and 63.5 and 63.0 (C1, C2, C3 of glycerol, $POCH_2CH_2N$), 53.1, 53.0, 52.6, 52.4, 52.3, 48.00 (MeOH), 42.5, 42.4, 42.3, 40.8, 40.0, 38.5, 35.4, 35.2, 40.0, 33.9, 31.9, 29.7, 29.5, 29.3, 29.2, 28.9, 27.1, 22.6, 13.8 (2 CH_3) ppm. HRESIMS (Figure S3), *m/z*: found 665.9954; calc. for $C_{87}H_{145}N_{18}O_{33}P$ $[M-3H]^{3-}$ 665.9914.

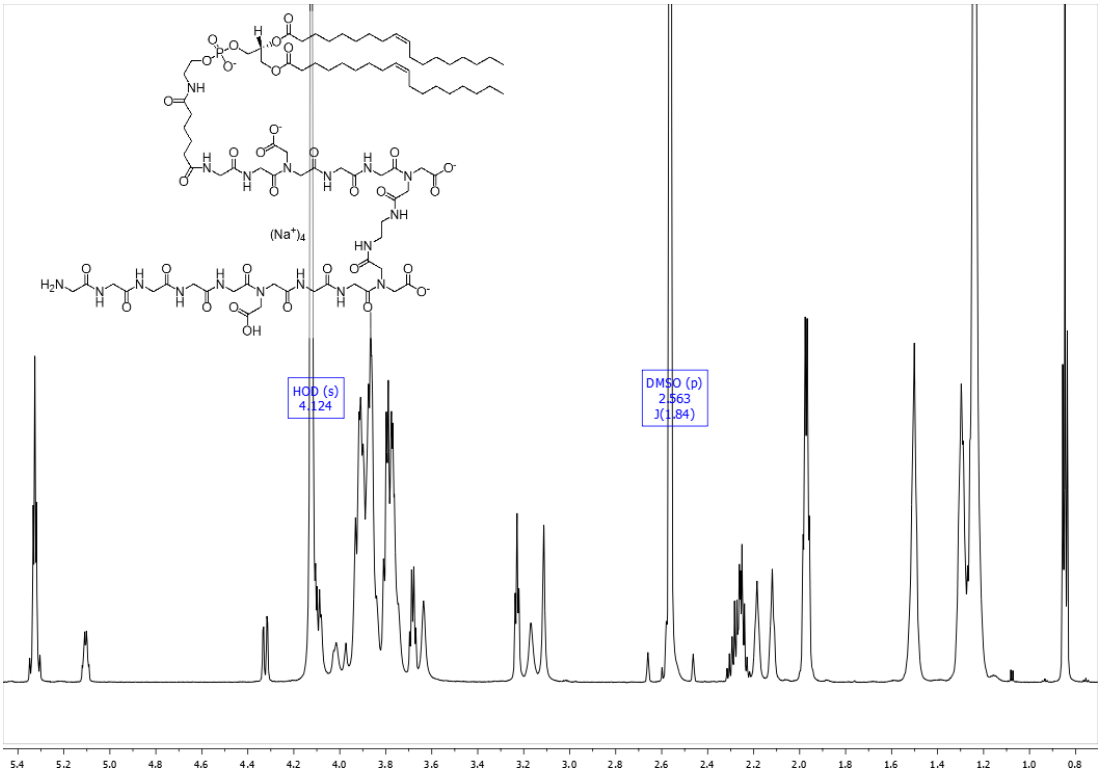


Figure S1 ^{1}H NMR spectrum of H_2N-Gly_3-CMG(2)-DOPE (D_6-DMSO/D_2O 3:1, 303K, 700 MHz).

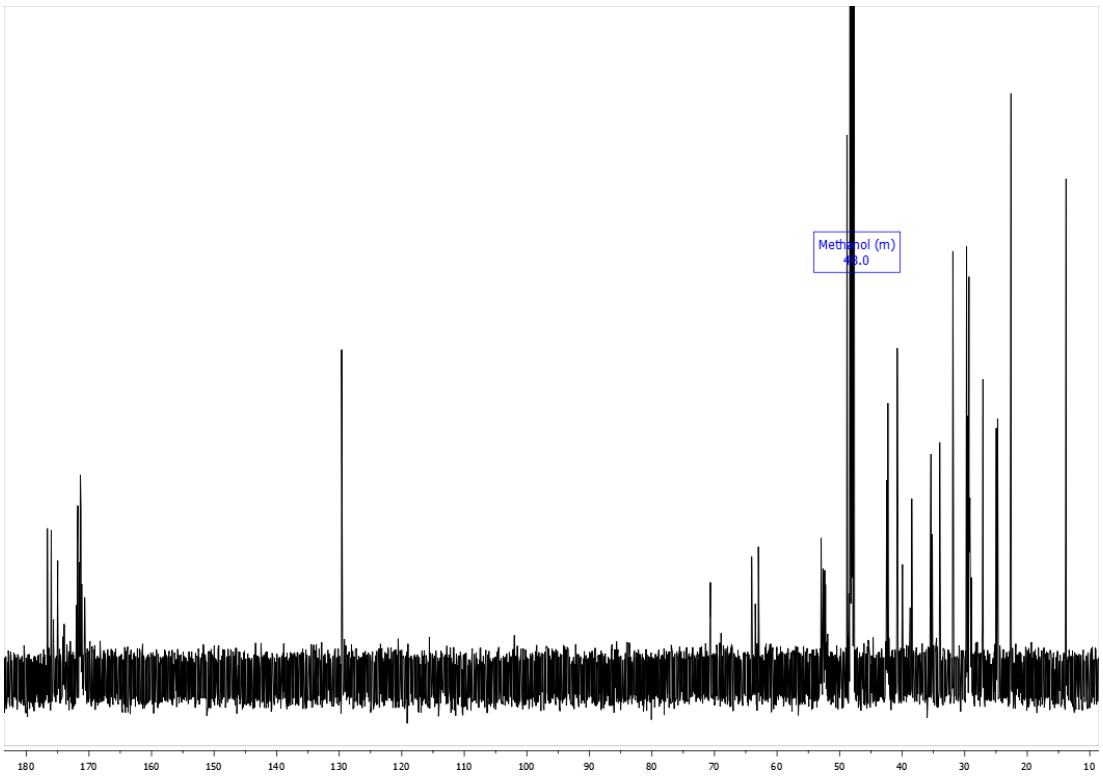


Figure S2 ^{13}C NMR spectrum of H_2N-Gly_3-CMG(2)-DOPE (D_2O/CD_3OD 2:1, 303K, 201 MHz).

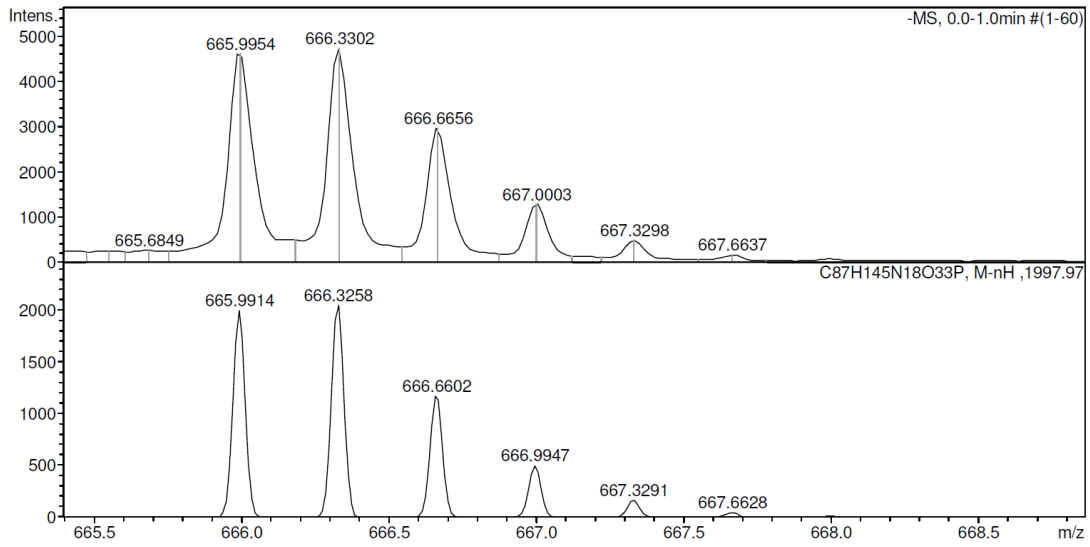


Figure S3 HRESIMS of H_2N-Gly_3-CMG(2)-DOPE: found m/z 665.9954; calc. for $C_{87}H_{145}N_{18}O_{33}P$ $[M-3H]^{3-}$ 665.9914.

3. Production of recombinant proteins

3.1 Preparation of recombinant protein A: ProtA-LPETG-His_6 (ProtA)

The construct *[leader peptide]~[domain B of protein A]~[domain B of protein A]~LPETGEAHHHHHHGC* was used. It includes a leader sequence, two copies of domain B of protein A, an LPETG peptide specific for enzyme recognition, and a His tag to facilitate isolation of the construct. The producer strain BL21(DE3) pLysS/pET32a-A-LPETG-His_6 was obtained by transforming *Escherichia coli* BL21(DE3) pLysS cells with the expression plasmid DNA pET32a-A-LPETG-His_6. A detailed description can be found in the patent[S2] and paper.[S3]

3.2 Preparation of recombinant protein mCherry: mCherry-LPETG-His_6 (mCherry)

The construct *[leader peptide]~[mCherry]~LPETGLEHHHHHH* was used. The production of the recombinant mCherry-LPETG-His_6 protein included obtaining a plasmid for expression of the recombinant protein, selecting an *Escherichia coli* strain capable of producing the mCherry-LPETG-His_6 protein, and isolating the protein. To create a strain producing the hybrid protein mCherry-LPETG-His_6, consisting of the red fluorescent protein mCherry, a motif for sortase recognition and a hexahistidine sequence at the C-terminus, an expression plasmid vector based on the pET22 vector was created, and the synthetic DNA sequence encoding the fusion protein was constructed. *Escherichia coli* BL21(DE3) pLysS cells were transformed with the pet22b-mCherry-Sort-HS expression vector. After induction, bacterial cells (35 g) were separated by centrifugation (5000 × g, 20 min, 4 °C) and lysed using an ultrasonic disintegrator (Elma, Schmidbauer GmbH, Singen, Germany) in 350 mL of buffer A (50 mM Tris·HCl, 1 M NaCl, 1 mM PMSF, 5 mM EDTA, pH 7.5). The resulting protein from the supernatant after centrifugation was separated on an XK 26/20 column (Cytiva, Life Sciences, Marlborough, MA, USA) packed with Ni-IMAC SepFast metal chelate resin (BioToolomics, ConestCo., Durham DH8 6 TJ, USA) under gradient elution conditions of 0→0.25 M imidazole in buffer B (50 mM Tris·HCl, 0.5 M NaCl, pH 8). At the next stage, an XK 26/20 column (Cytiva, Life-Sciences,

USA) packed with Q-Sepharose FF sorbent (GE Healthcare, Uppsala, Sweden) pre-equilibrated with buffer C (50 mM Tris·HCl, pH 8.0) was used. The protein was eluted with 0→0.5 M NaCl gradient in buffer C. At the final stage, the protein solution was desalted on a Sephadex G-25 XK 50/100 column (Cytiva, Life-Sciences, USA) using buffer C to yield 350 mg of mCherry-LPETG-His_6 protein with an electrophoretic purity >90%.

3.3. Preparation of recombinant sortase A (SrtA)

Recombinant Ca-independent SrtA was prepared by the method described previously.[S4,S5] To obtain a SrtA producing strain, the expression plasmid DNA rET21a-Sort6 was constructed on the basis of the plasmid vector rET21a and a synthetic DNA sequence encoding the SrtA with a C-terminal hexahistidine tag. The expression vector pet21b-Sort6 was used for transformation of *Escherichia coli* BL21(DE3) pLysS cells. Briefly, after the induction of SrtA expression, the bacterial pellet (50 g) was separated by centrifugation (5000 × *g*, 20 min, 4 °C) and lysed using an ultrasonic disintegrator (Elma, Schmidbauer GmbH, Singen, Germany) in buffer D (50 mM Tris, 0.5 M NaCl, 10% glycerol, pH 7.5). The resulting protein from the supernatant after centrifugation was applied to an XK 26/20 column packed with Ni-IMAC metal chelate resin (SepFast BioToolomics, Consett Co., Durham DH8 6TJ, United Kingdom), which was pre-equilibrated with buffer D containing 20 mM imidazole. After the sample application, the column was washed with buffer D containing 50 mM imidazole, and then the target protein was eluted with buffer D containing 500 mM imidazole. Finally, the SrtA solution was purified by size exclusion chromatography on a Sephadex G-25 XK 50/100 column (Cytiva, Life-Sciences, USA), elution with buffer E (50 mM Tris, 150 mM NaCl, 10% glycerol, pH 7.0) to yield 550 mg of SrtA with an electrophoretic purity >90%.

3.4 Concentration of proteins

The initial concentration of proteins was determined spectrophotometrically using the extinction coefficient (ε) calculated in the ProtParam tool software (ProtA-LPETG-His_6 protein, ε_{280} = 4470 $M^{-1}cm^{-1}$; mCherry-LPETG-His_6 protein, ε_{280} = 39880 $M^{-1}cm^{-1}$; SrtA, ε_{280} =17420 $M^{-1}cm^{-1}$).

4. General protocol for coupling CMG-armed lipid with proteins

To 0.43 mL of a 150 μM solution of mCherry-LPETG-His_6 in buffer C was added 0.052 mL of a 12.5 mM solution of H_2N-G_3-CMG(2)-DOPE in the same buffer, and the mixture was diluted with 0.24 mL of buffer C. Then 0.28 mL of a solution of 77 μM SrtA in buffer E was added and the mixture was incubated at 37 °C for 3 h. Imidazole (2.72 mg) and NaCl (53 mg) were then added with stirring, and the resulting solution was applied to a C10/10 column (GE Healthcare Life Sciences, Germany) packed with 1 mL of Ni-IMAC SepFast metal chelate resin (BioToolomics, Consett Co., Durham DH8 6TJ, UK) equilibrated with buffer F (50 mM Tris·HCl, 1M NaCl, 40 mM imidazole, pH 8.0). The column was washed with the same buffer until the baseline was reached (UV control), the wash-through (2.5 mL) containing the target conjugate was two-fold diluted with buffer C and applied to a C10/10 column (GE Healthcare Life Sciences, Germany) packed with 2 mL of TOYOPEARL Butyl resin (TOSOH, Japan) equilibrated with buffer B. The column was rinsed with 8 mL of buffer C, and mCherry LPET-G_3-CMG(2)-DOPE was eluted with the same buffer containing 20% of ethanol. Ethanol was removed *in vacuo* at 35 °C, the solution was filtered (Spin-X® Centrifugal Tube Filters 0.22 μm, Corning, USA) and used further as is. The concentration and the yield of the target mCherry LPET-G3-CMG(2)-DOPE were estimated using UV-Vis spectroscopy (mCherry, absorbance at 586 nm, ε = 88000 M^{-1} cm^{-1}, yield 19%).

The ProtA conjugate, ProtA-LPET-G_3-CMG(2)-DOPE, was synthesized similarly except for the final stage, as follows. Purified material after evaporation was applied to a C 10/40

column (GE Healthcare Life Sciences, Germany) packed with Sephadex G-25 (Cytiva, Life-Sciences, USA) equilibrated with 10 mM ammonium bicarbonate. The target conjugate was collected and freeze-dried using a FreeZone 2.5 Benchtop Freeze Dryer (LABCONCO, USA) to give ProtA-LPET-G_3-CMG(2)-DOPE in 64% yield. The lipid conjugates obtained were analyzed by SDS-PAGE and HPLC.

5. Monitoring the lipidation reaction of the mCherry protein using SDS gel electrophoresis (Figure S4) and HPLC (Figure S5)

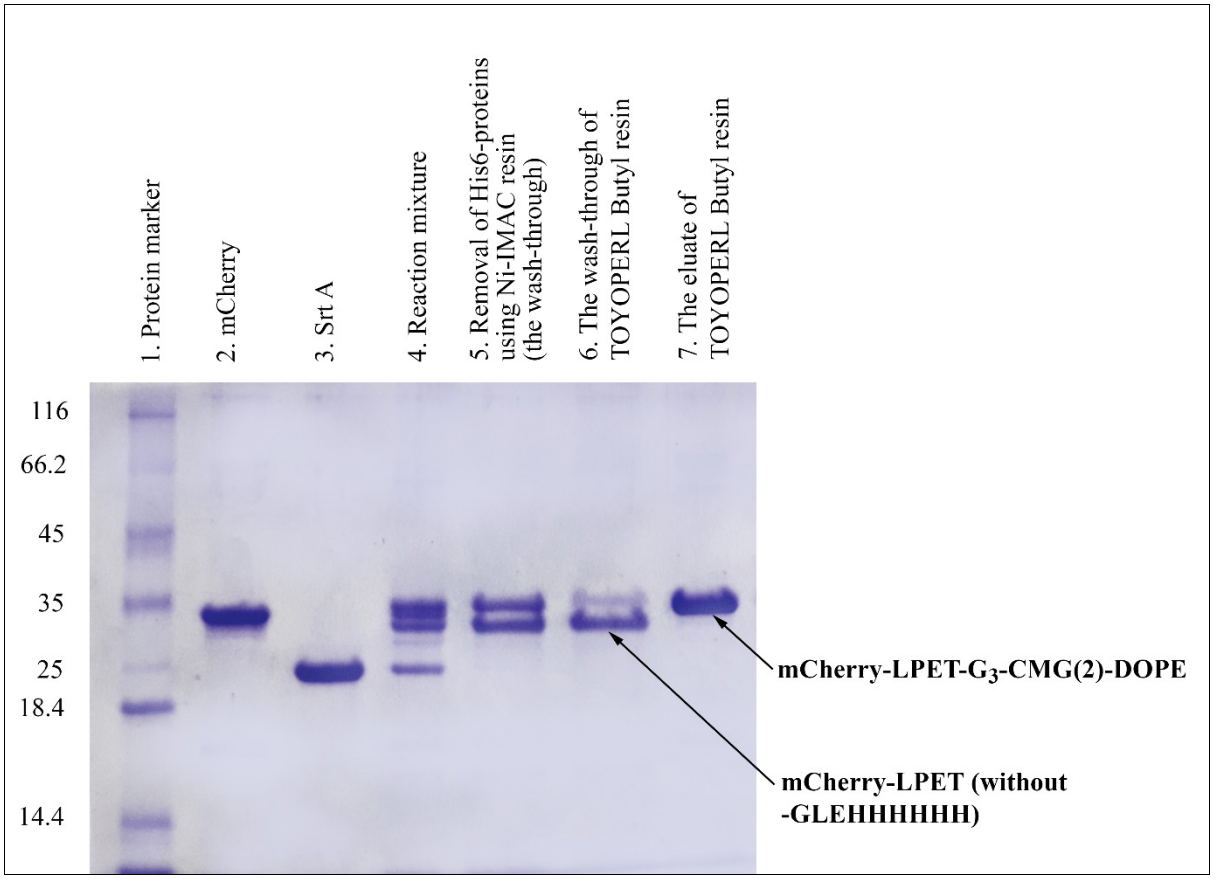


Figure S4 SDS-PAGE analysis of lipid-modified mCherry.

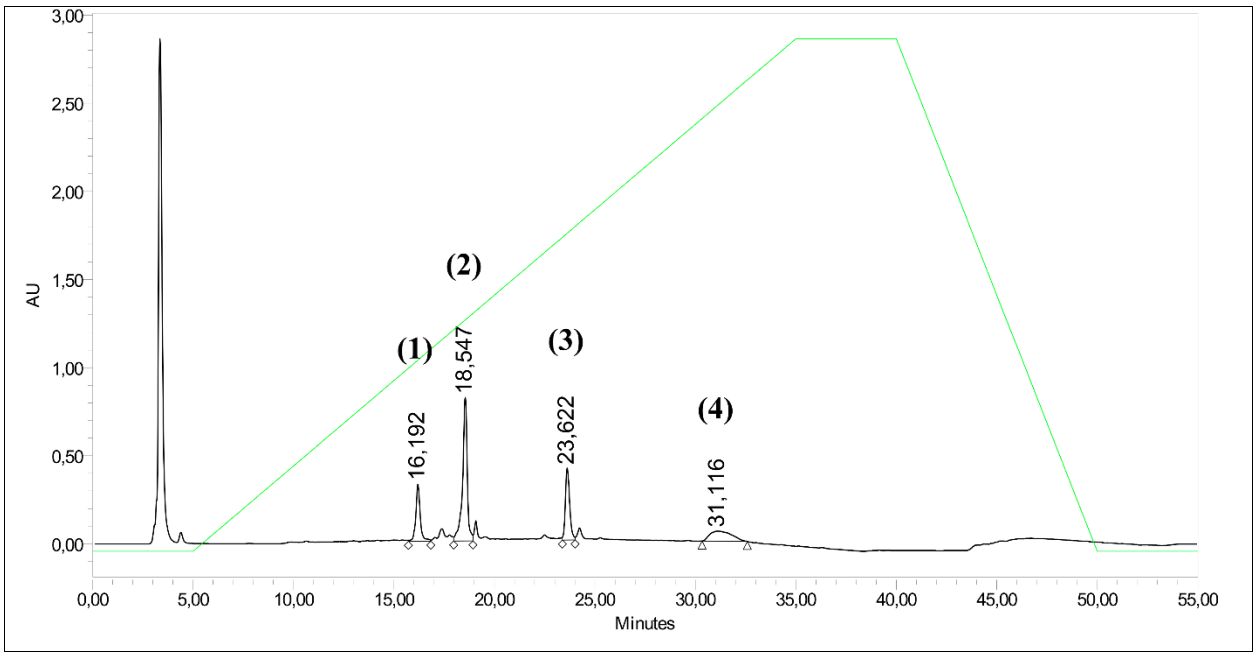


Figure S5 HPLC-profiles of the reaction mixture for the synthesis of the mCherry-G_3-CMG(2)-DOPE; 3 h, 37 °C, detection at 214 nm. (1) Srt A; (2) mCherry-LPET (without -GLEHHHHHH; (3) mCherry-LPET-G_3-CMG(2)-DOPE; (4) H_2N-G_3-CMG(2)-DOPE.

6. High-performance liquid chromatography

HPLC was performed on the Waters system (1525 Binary HPLC Pump, 2489 UV/VIS Detector, Breeze v2 software, Waters Inc., Milford, MA, USA) using a Protein C4 (4.6×250 mm) column (GRACE VYDAC, USA). Eluent A: acetonitrile 5%+water 95%+0.1% TFA. Eluent B: 95% acetonitrile+5% water+0.1% TFA, injection 10 μL, detection at 214 nm and 280 nm. Gradient program: 0% B from 0 to 5 min, then 0→10% B from 5 to 35 min.

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