Supplementary Information

Chemogenetic Control of Protein Anchoring to Endomembranes in Living Cells with Lipid-Tethered Small Molecules

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Supplementary Figures

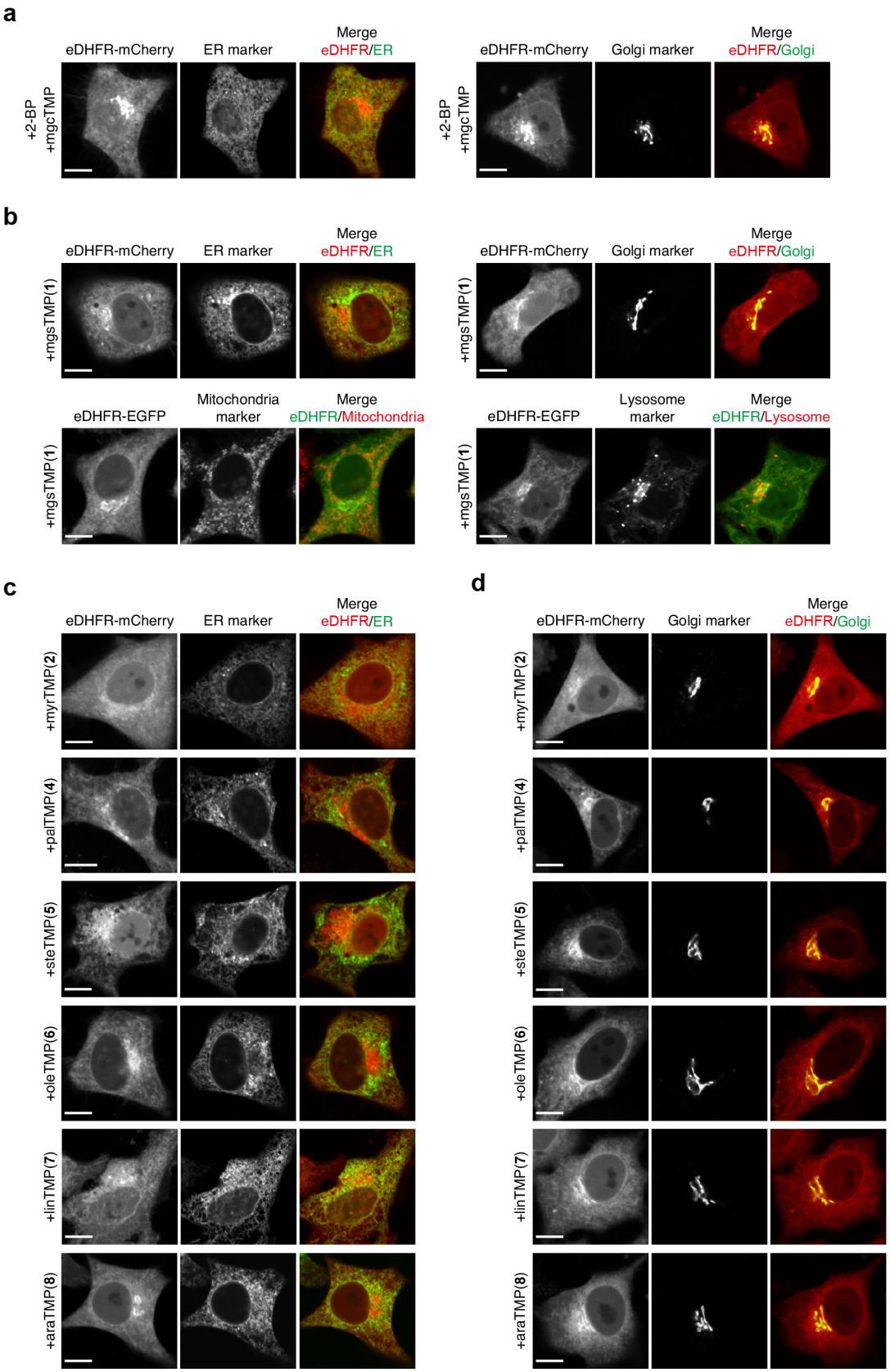

Figure S1. Colocalization analysis of SL-induced eDHFR-EGFP/mCherry translocation sites. The organelle markers used were ER-mAG1 (ER), AcGFP-Golgi (Golgi), MitoTracker Red (mitochondria), and LysoTracker Red DND99 (lysosome). (**a**) Confocal fluorescence images of a HeLa cell treated with mgcTMP under palmitoylation inhibition. (**b**) Confocal fluorescence images of HeLa cells treated with unpalmitoylatable mgsTMP. The fluorescence signals of eDHFR-tagged protein merged well with those of the ER and Golgi makers, but not with those of the mitochondria and lysosome markers. (**c**, **d**) Confocal fluorescence images of HeLa cells expressing the ER (**c**) or Golgi marker (**d**) were taken after treatment with the indicated compound. The fluorescence signals of eDHFR-mCherry merged well with those of the ER and Golgi makers. Scale bars, 10 μm.

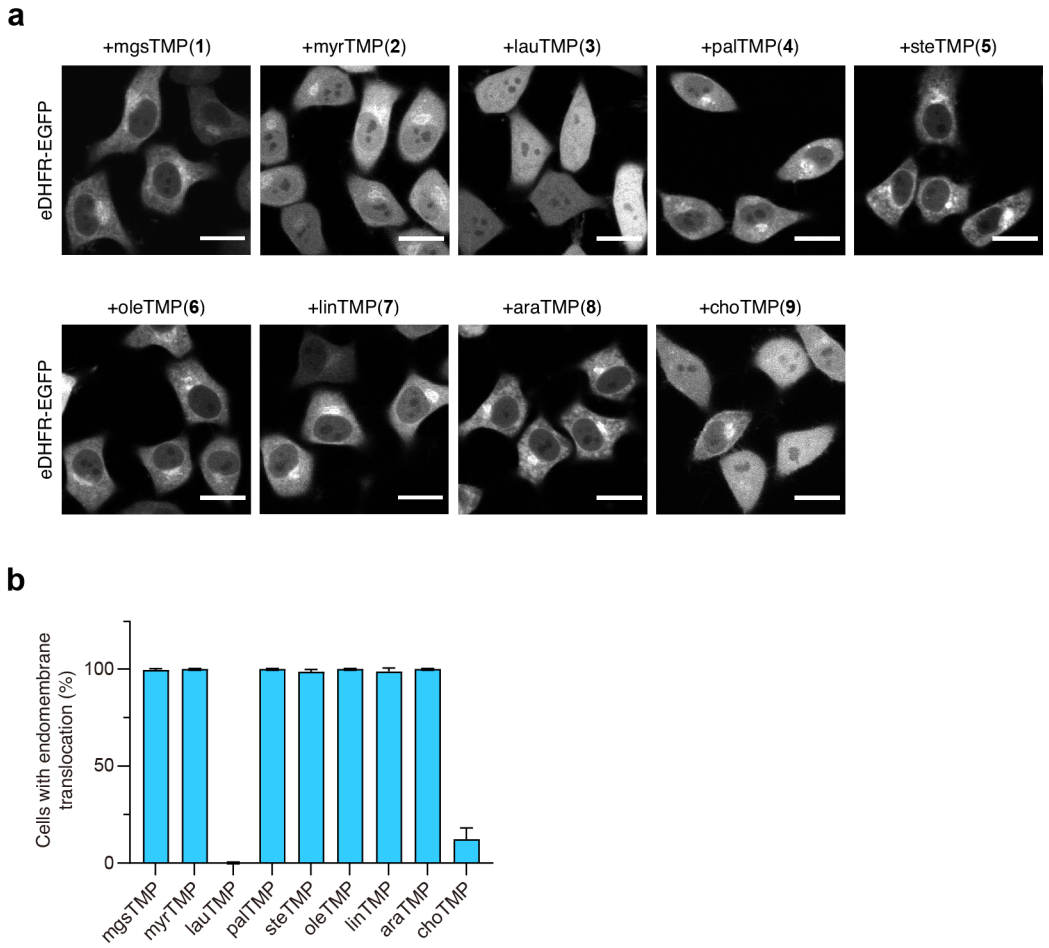

Figure S2. Evaluation of protein translocation efficiency of lipid-tethered TMPs. (**a**) HeLa cells expressing eDHFR-EGFP were imaged after treatment with the indicated compound (10 μM) for 1 h. (**b**) Quantification of cells with endomembrane translocation of eDHFR-EGFP after treatment with lipid-tethered TMPs **1**–**9**. Blue bars indicate the percentage of cells showing endomembrane localization of eDHFR-EGFP. Data are represented as the mean ± SD (n > 300 cells). Scale bars, 20 μm.

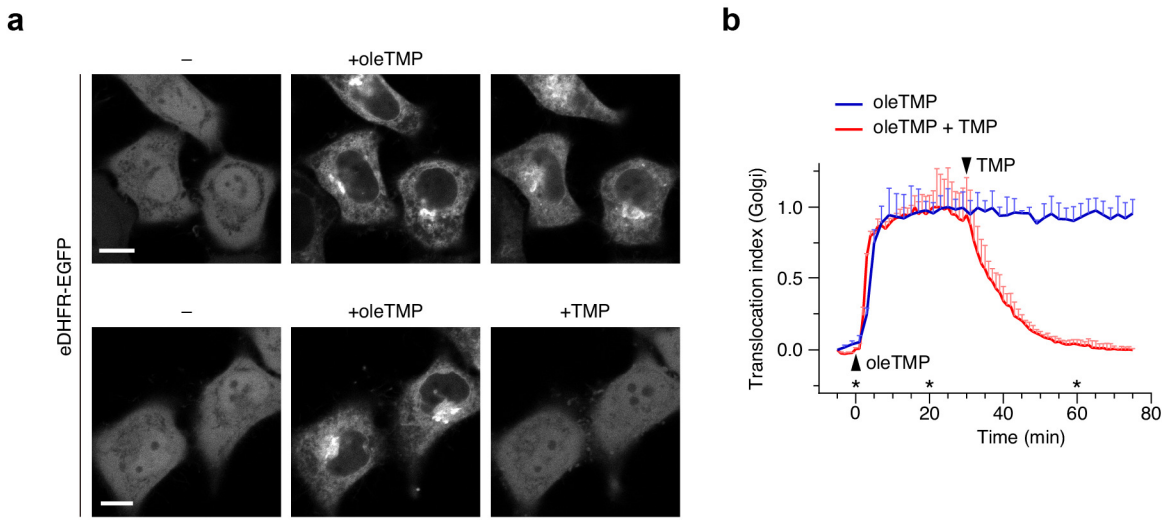

Figure S3. Reversible protein anchoring to endomembranes. (**a**) (Top) Sustained endomembrane anchoring of eDHFR-EGFP by oleTMP addition only. (Bottom) Reversible endomembrane anchoring of eDHFR-EGFP by stepwise addition of oleTMP and TMP. Confocal fluorescence images of HeLa cells expressing eDHFR-EGFP were taken at the time points indicated by asterisks in **b**: before (left), 20 min after the addition of 5 μM oleTMP (center), and 30 min after the subsequent addition of 50 μM TMP (right). oleTMP and TMP were added at 0 and 30 min, respectively. (**b**) Time course of endomembrane anchoring of eDHFR-EGFP. The normalized ratios of the Golgi to the nucleus fluorescence intensity are plotted as a function of time. Data are represented as the mean ± SD (n = 3 cells). Scale bars, 10 μm.

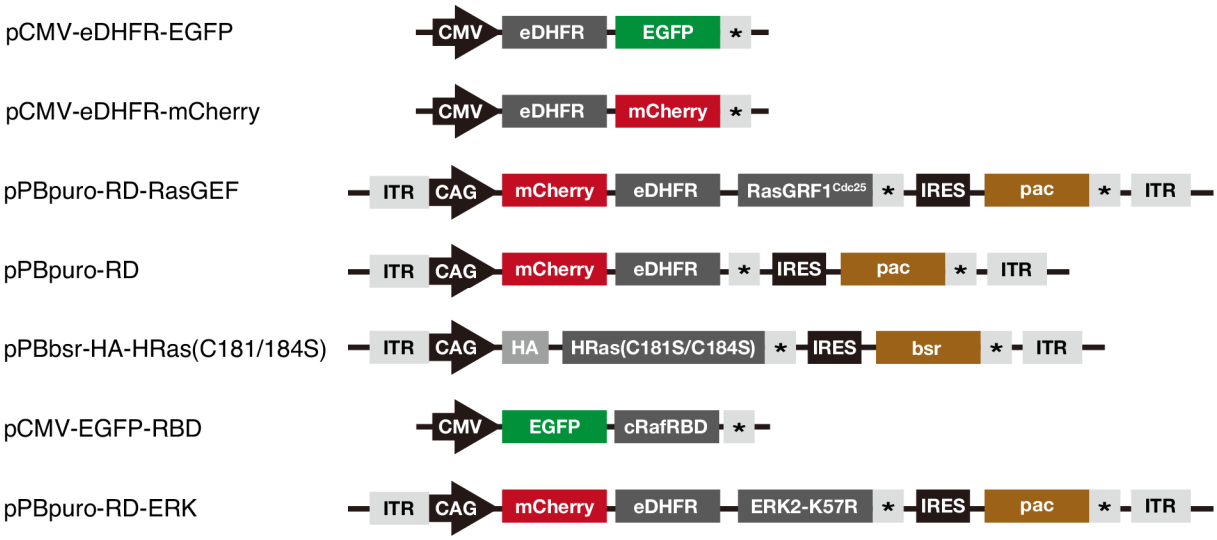

Figure S4. Schematic representations of domain structures of constructs used in this study.

a

pCMV-eDHFR-EGFP

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b

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e

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g

pPBpuro-RD-ERK

>Amino acid sequence

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Figure S5. DNA and amino acid sequences of constructs used in this study. (**a**) pCMV-eDHFR-EGFP: grey, eDHFR; green, EGFP. (**b**) pCMV-eDHFR-mCherry: grey, eDHFR; red, mCherry. (**c**) pPBpuro-RD-RasGEF: red, mCherry; grey, eDHFR; purple, a RasGEF domain (residues 1018–1273) from human RasGRF1; yellow marker, an internal ribosomal entry site from Encephalomyocarditis virus (EMCV IRES)[S1]; brown, puromycin *N*-acetyltransferase (pac). (**d**) pPBpuro-RD: red, mCherry; grey, eDHFR; yellow marker, EMCV IRES; brown, pac. (**e**) pPBbsr-HA-HRas(C181/184S): blue, HA-tag; grey, endomembrane-targeted HRas [human HRas(C181S/C184S)[S2]]; yellow marker, EMCV IRES; brown, blasticidin *S*-deaminase (bsr). (**f**) pCMV-EGFP-RBD: green, EGFP; grey, a Ras-binding domain (residues 51–131) from human cRaf[S2]. (**g**) pPBpuro-RD-ERK: red, mCherry; grey, eDHFR; blue, *Xenopus laevis* ERK2-K57R (kinase-dead mutant)[S3,S4]; yellow marker, EMCV IRES; brown, pac.

Supplementary Movies

Move S1. palTMP-induced endomembrane anchoring of eDHFR-EGFP in HeLa cells [time-lapse movie of **Figure 2c** (top)]. Scale bar, 20 μm.

Movie S2. oleTMP-induced endomembrane anchoring of eDHFR-EGFP in HeLa cells [time-lapse movie of **Figure 2c** (bottom)]. Scale bar, 20 μm.

Movie S3. Reversible translocation of eDHFR-EGFP in HeLa cells by stepwise addition of oleTMP and TMP [time-lapse movie of **Figure S3a** (bottom)]. Scale bar, 20 μm.

Movie S4. Endomembrane Ras activation by oleTMP-induced endomembrane recruitment of RD-RasGEF (time-lapse movie of **Figure 3b**). Scale bar, 10 μm.

Movie S5. Endomembrane trapping of RD-ERK by oleTMP and its release by TMP [time-lapse movie of **Figure 4b** (bottom)]. Scale bar, 20 μm.

Supplementary Methods: Chemical Synthesis

General materials and methods.

All chemical reagents and solvents were purchased from commercial suppliers (Watanabe Chemical Industries, Tokyo Chemical Industry, FUJIFILM Wako Pure Chemical Corp., and Kanto Chemical) and used without further purification. Reverse-phase HPLC was performed on a Hitachi LaChrom Elite system with UV detection at 220 nm using a YMC-Pack ODS-A column (10 × 250 mm or 20 × 250 mm) or a YMC-Pack C4 column (10 × 250 mm). ^{1}H NMR spectra were recorded on a JEOL AL-400 (400 MHz) or Bruker AVANCE III HD400SJ (400 MHz) spectrometer. ^{1}H NMR chemical shifts were referenced to tetramethylsilane (0 ppm). High-resolution mass spectra were measured on a Thermo Scientific Extractive Plus Orbitrap mass spectrometer.

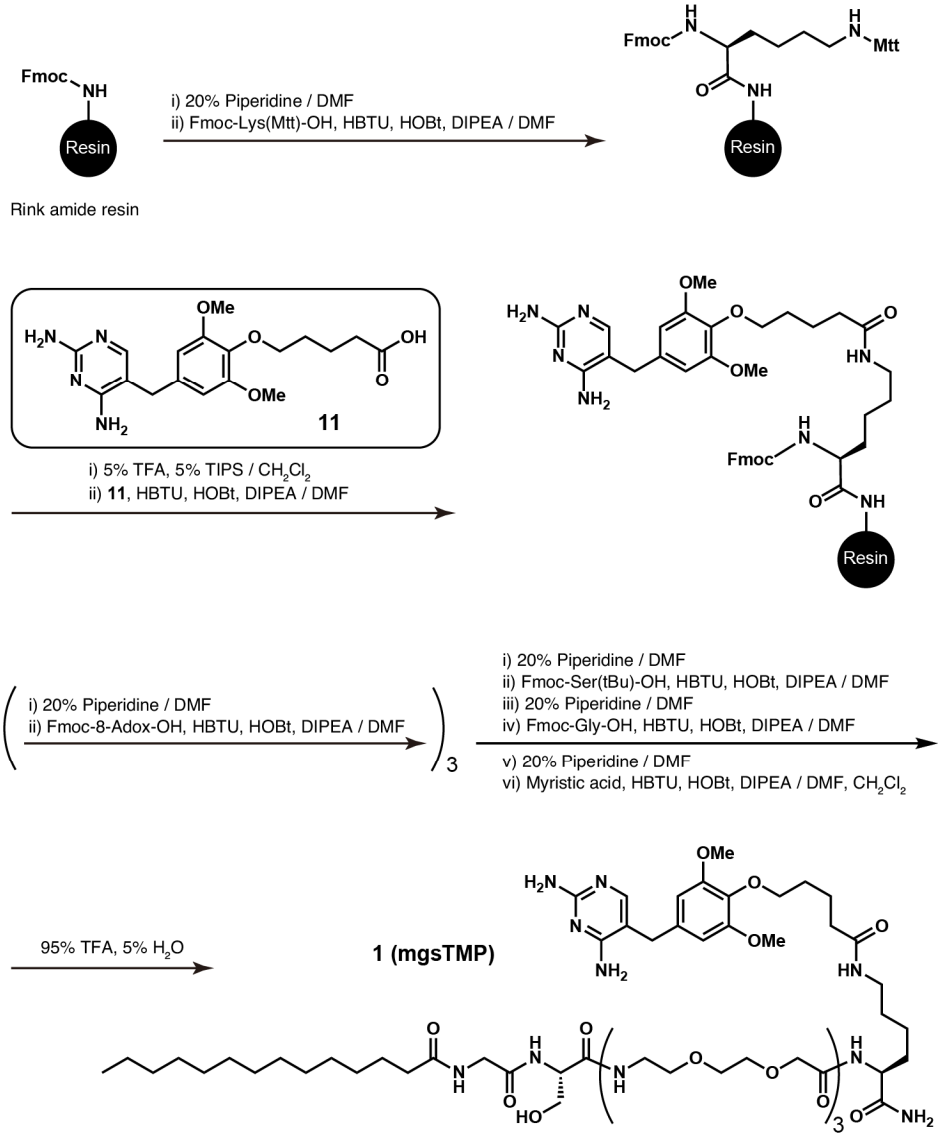
Reagent abbreviations

DIPEA: *N*,*N*-diisopropylethylamine
DMF: *N*,*N*-dimethylformamide
Fmoc-Adox-OH: Fmoc-8-amino-3,6-dioxaoctanoic acid
HBTU: *O*-(benzotriazole-1-yl)-*N*,*N*,*N*',*N*'-tetramethyluronium hexafluorophosphate
HOBt: 1-hydroxybenzotriazole (monohydrate)
TFA: trifluoroacetic acid
TIPS: triisopropylsilane
TMS: tetramethylsilane

General methods for solid-phase synthesis.

Compounds **1-10** were synthesized manually on Rink amide resin or Sieber amide resin by standard Fmoc-based solid-phase peptide synthesis protocols. Fmoc deprotection was performed with 20% piperidine in DMF at room temperature for 15 min. Amino acid coupling reactions were performed at room temperature with a mixture of Fmoc-protected amino acid (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. All Fmoc deprotection and coupling steps were monitored by the Kaiser test.[S5] Unless otherwise stated, all washing procedures were performed with DMF. Compound **11** was synthesized as described previously.[S6]

Synthesis of Compound 1 (mgsTMP)



Scheme S1. Synthetic route of **1** (mgsTMP)

Compound **1** (mgsTMP) was synthesized on Rink amide resin (0.65 mmol/g) (62 mg, 40 μmol). First, Fmoc-Lys(Mtt)-OH was coupled to the resin. After washing the resin with DMF, MeOH, and CH_2Cl_2, the Mtt group was deprotected by treatment with CH_2Cl_2 containing 5% TFA and 5% TIPS. The resin was then washed with CH_2Cl_2, MeOH, and DMF. **11** was coupled to the side chain of the lysine with a mixture of **11** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. Subsequently, Fmoc deprotection and coupling reactions were repeated using Fmoc-Adox-OH, Fmoc-Ser(tBu), and Fmoc-Gly-OH as building blocks. The N-terminus was myristoylated using a mixture of

myristic acid (4.1 eq.), HBTU (4.0 eq.), HOBt (4.0eq.), and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing with DMF, MeOH, and CH_2Cl_2, the resin was dried *in vacuo*. Deprotection and cleavage from the resin was performed with 95% TFA containing 5% H_2O. The crude product was precipitated by Et_2O and purified by reversed-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **1** as a white solid [20 mg, 30% (as a mono-TFA salt)].

Compound **1** (mgsTMP)

^{1}H NMR (400 MHz, CD_3OD): δ 7.24 (1H, s), 6.56 (2H, s), 4.60 (1H, m), 4.41 (1H, m), 4.05–3.96 (6H, m), 3.89 (4H, m), 3.79 (8H, m), 3.66 (12H, m), 3.58 (6H, m), 3.45 (4H, m), 3.41 (2H, m), 3.18 (2H, t, J = 6.0 Hz), 2.25 (4H, m), 1.99–1.34 (12H, m), 1.28 (22H, m), 0.89 (3H, t, J = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1293.7664; found, 1293.7638.

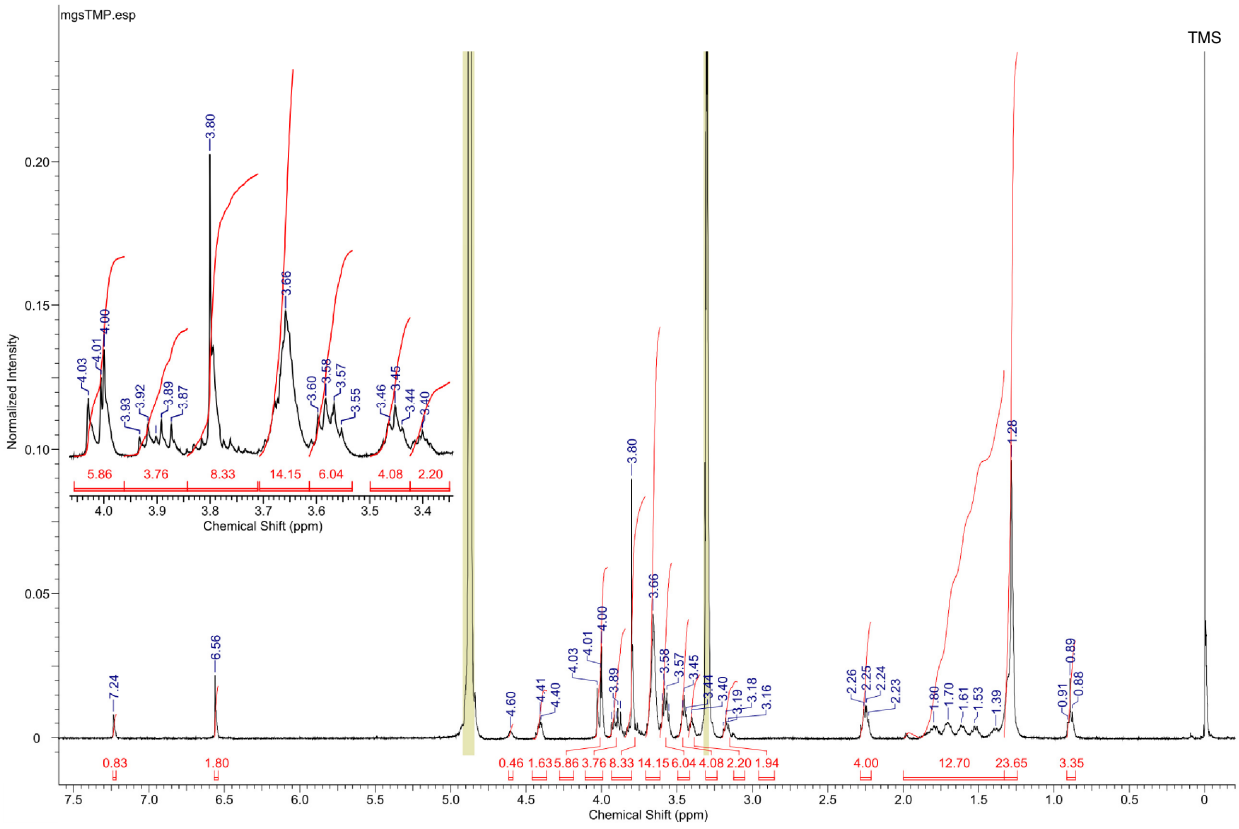
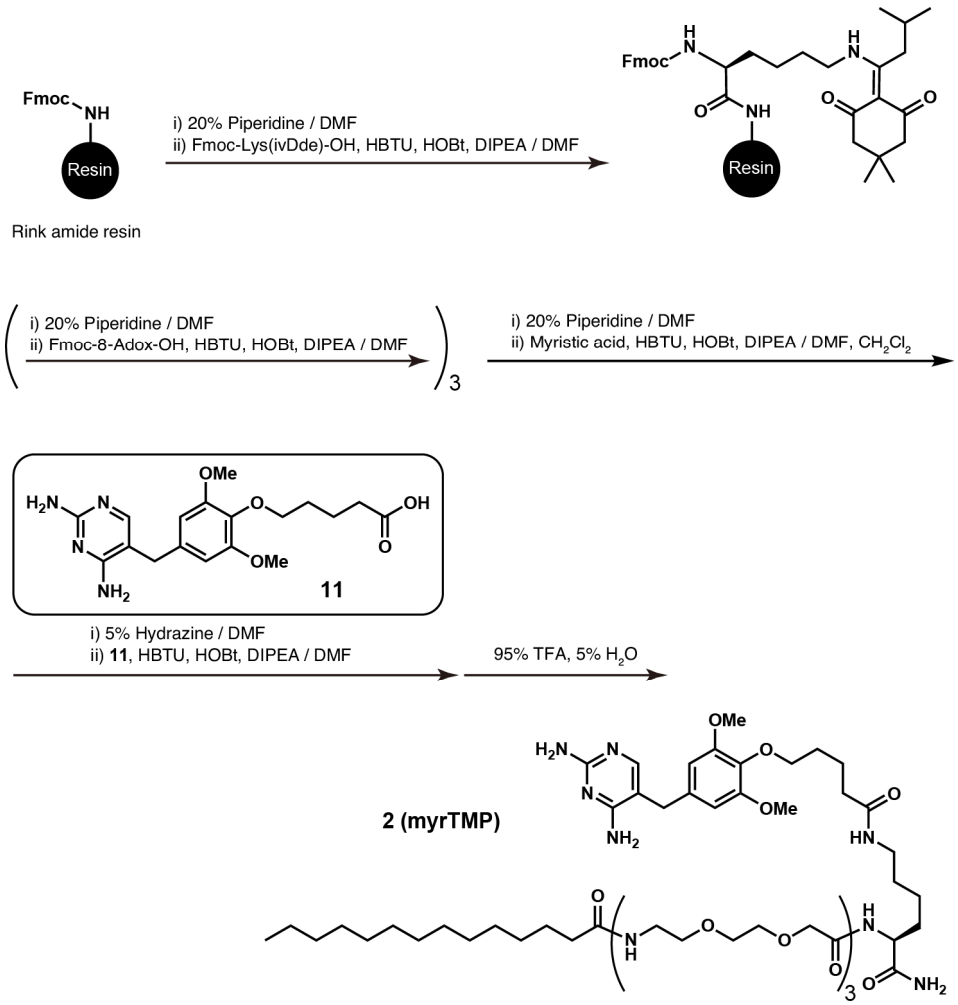


Figure S6. ^{1}H NMR spectrum of **1** (mgsTMP) in CD_3OD.

Synthesis of Compound 2 (myrTMP)



Scheme S2. Synthetic route of **2** (myrTMP)

Compound **2** (myrTMP) was synthesized on Rink amide resin (0.39 mmol/g) (130 mg, 51 μmol). First, Fmoc-Lys(ivDde)-OH and Fmoc-Adox-OH (x3) were coupled to the resin. The N-terminus was then myristoylated using a mixture of myristic acid (4.1 eq.), HBTU (4.0 eq.), HOBt (4.0 eq.), and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing the resin with DMF, the ivDde group was deprotected by treatment with DMF containing 5% hydrazine monohydrate. The resin was then washed with DMF. **11** was coupled to the side chain of the lysine with a mixture of **11** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing with DMF, MeOH, and CH_2Cl_2, the resin was dried *in vacuo*. Cleavage from the resin was performed with 95% TFA containing 5% H_2O. The crude product was purified by reverse-phase HPLC using

a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **2** (myrTMP) as a white solid [26.7 mg, 42% (as a mono-TFA salt)].

Compound **2** (myrTMP)

^{1}H NMR (400 MHz, CD_3OD): δ 7.23 (1H, s), 6.56 (2H, s), 4.41 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.92 (2H, m), 3.80 (6H, s), 3.66 (12H, m), 3.58 (4H, m), 3.55 (2H, m), 3.45 (4H, m), 3.37 (2H, t, J = 6.0 Hz), 3.18 (2H, t, J = 6.0 Hz), 2.25 (2H, t, J = 8.0 Hz), 2.19 (2H, t, J = 8.0 Hz), 1.91–1.35 (12H, m), 1.28 (22H, m), 0.89 (3H, t, J = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1149.7129; found, 1149.7090.

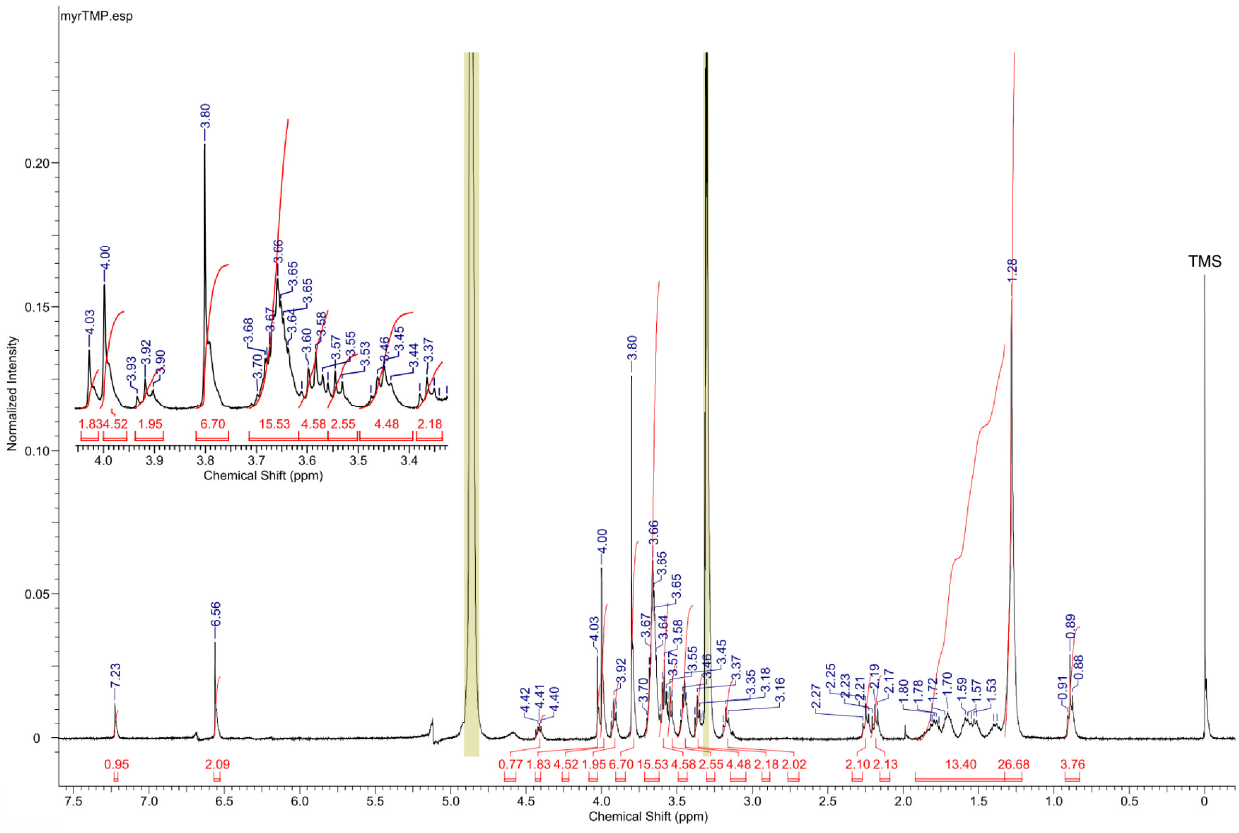
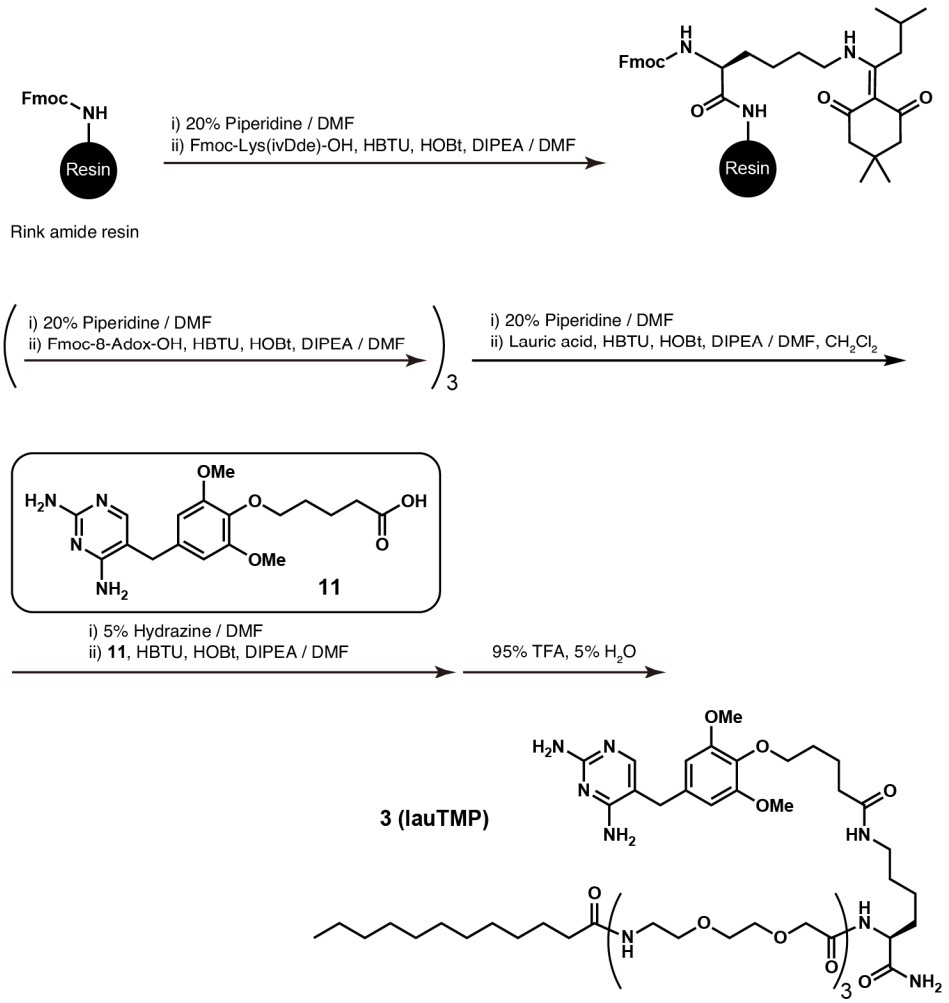


Figure S7. ^{1}H NMR spectrum of **2** (myrTMP) in CD_3OD.

Synthesis of Compound 3 (lauTMP)



Scheme S3. Synthetic route of **3** (lauTMP)

Compound **3** (lauTMP) was synthesized on Rink amide resin (0.58 mmol/g) (35 mg, 20 μmol). First, Fmoc-Lys(ivDde)-OH and Fmoc-Adox-OH (x3) were coupled to the resin. The N-terminus was lipidated using a mixture of lauric acid (4.1 eq.), HBTU (4.0 eq.), HOBt (4.0 eq.), and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing the resin with DMF, the ivDde group was deprotected by treatment with DMF containing 5% hydrazine monohydrate. The resin was then washed with DMF. **11** was coupled to the side chain of the lysine with a mixture of **11** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing with DMF, MeOH, and CH_2Cl_2, the resin was dried *in vacuo*. Cleavage from the resin was performed with 95% TFA containing 5% H_2O. The crude product was purified by reverse-phase HPLC using a semi-preparative C18 column

(a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **3** (lauTMP) as a white solid [9.6 mg, 39% (as a mono-TFA salt)].

Compound **3** (lauTMP)

^{1}H NMR (400 MHz, CD_3OD): δ 7.22 (1H, s), 6.56 (2H, s), 4.41 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.92 (2H, m), 3.80 (6H, s), 3.66 (12H, m), 3.58 (4H, m), 3.55 (2H, m), 3.45 (4H, m), 3.37 (2H, t, J = 6.0 Hz), 3.18 (2H, t, J = 6.0 Hz), 2.25 (2H, t, J = 8.0 Hz), 2.19 (2H, t, J = 8.0 Hz), 1.91–1.35 (12H, m), 1.28 (18H, m), 0.89 (3H, t, J = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1121.6816; found, 1121.6765.

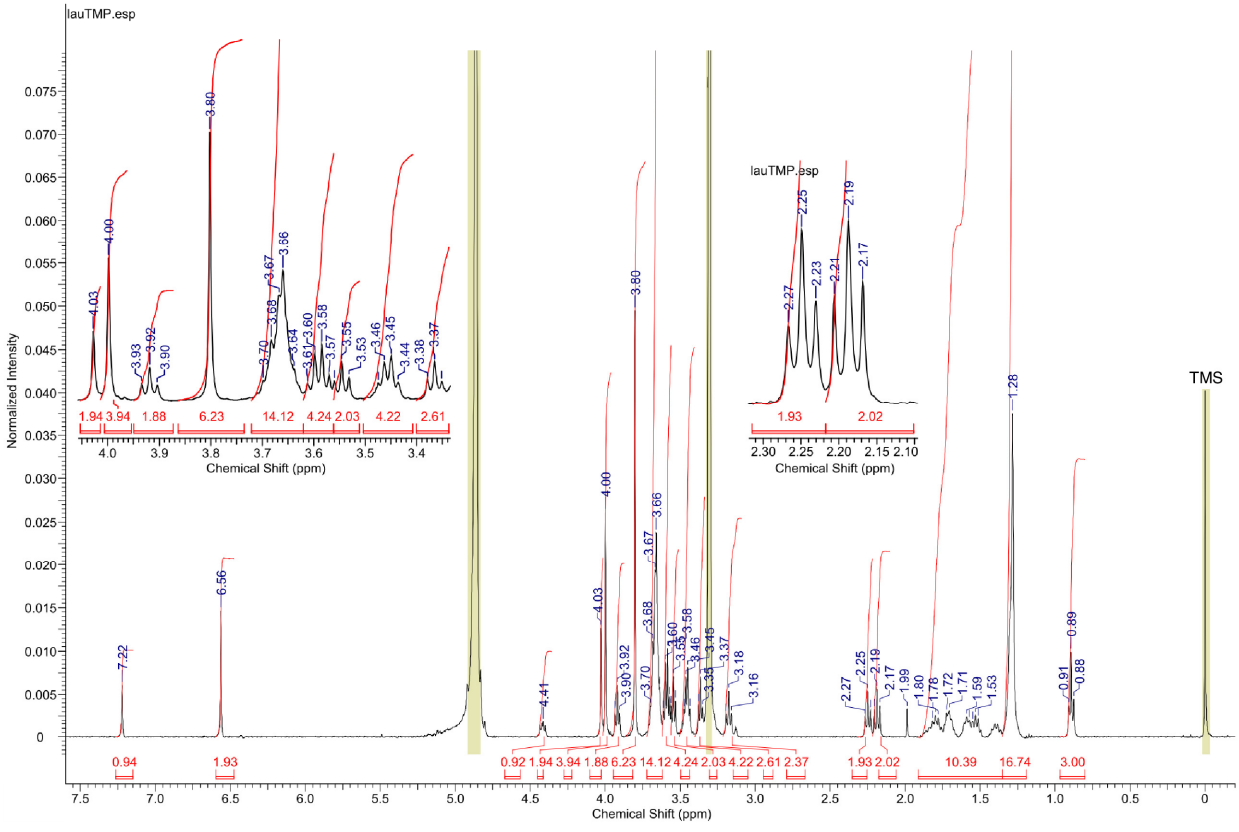
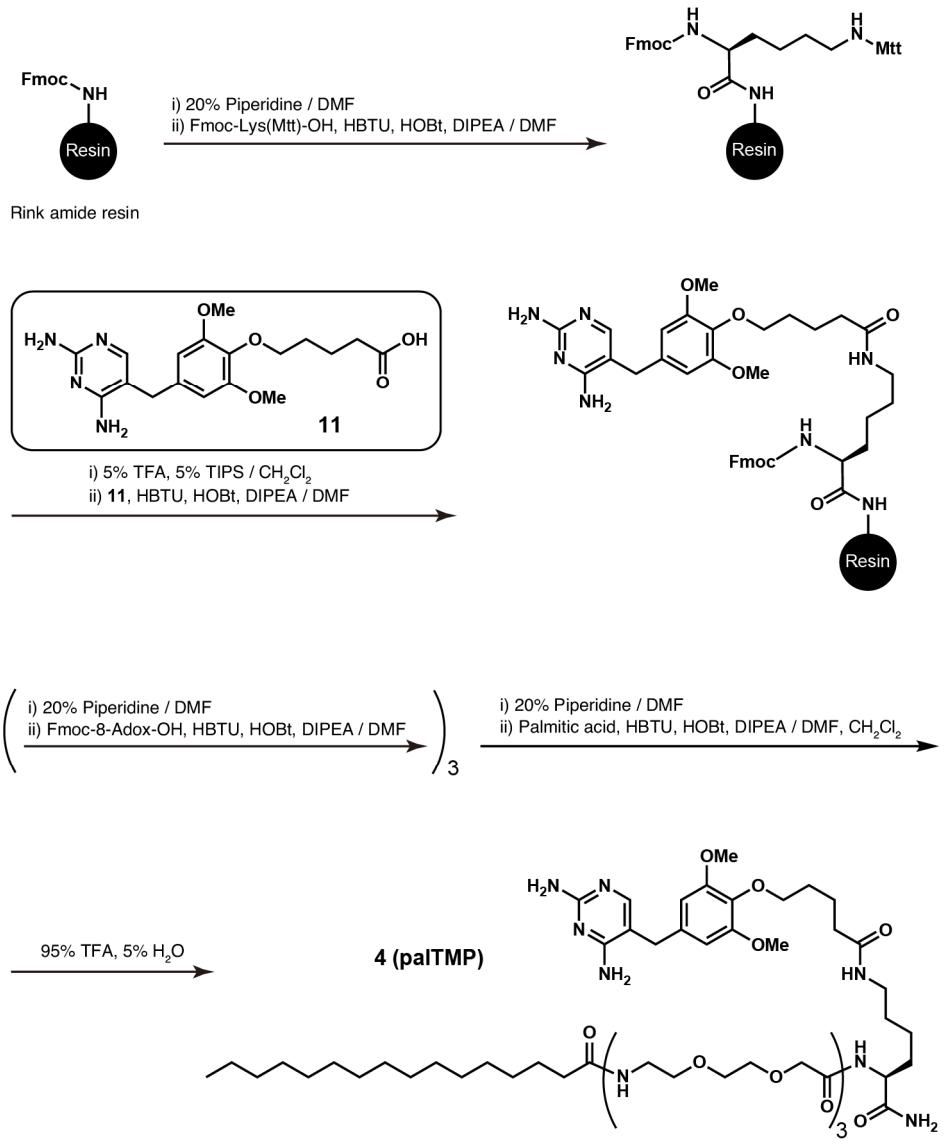


Figure S8. ^{1}H NMR spectrum of **3** (lauTMP) in CD_3OD.

Synthesis of Compound 4 (palTMP)



Scheme S4. Synthetic route of **4** (palTMP)

Compound **4** (palTMP) was synthesized on Rink amide resin (0.55 mmol/g) (36 mg, 20 μmol). First, Fmoc-Lys(Mtt)-OH was coupled to the resin. After washing the resin with DMF, MeOH, and CH_2Cl_2, the Mtt group was deprotected by treatment with CH_2Cl_2 containing 5% TFA and 5% TIPS. The resin was then washed with CH_2Cl_2, MeOH, and DMF. **11** was coupled to the side chain of the lysine with a mixture of **11** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. Subsequently, Fmoc deprotection and coupling reactions were repeated using Fmoc-Adox-OH as a building block. The N-terminus was palmitoylated using a mixture of palmitic acid (4.1 eq.), HBTU (4.0 eq.),

HOBt (4.0 eq.), and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing with DMF, MeOH, and CH_2Cl_2, the resin was dried *in vacuo*. Cleavage from the resin was performed with 95% TFA containing 5% H_2O. The crude product was purified by reverse-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **4** (palTMP) as a white solid [5.6 mg, 22% (as a mono-TFA salt)].

Compound **4** (palTMP)

^{1}H NMR (400 MHz, CD_3OD): δ 7.22 (1H, s), 6.56 (2H, s), 4.41 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.92 (2H, m), 3.80 (6H, s), 3.66 (12H, m), 3.58 (4H, m), 3.55 (2H, m), 3.45 (4H, m), 3.37 (2H, t, J = 6.0 Hz), 3.18 (2H, t, J = 6.0 Hz), 2.25 (2H, t, J = 8.0 Hz), 2.19 (2H, t, J = 8.0 Hz), 1.91–1.35 (12H, m), 1.28 (24H, m), 0.89 (3H, t, J = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1177.7442; found, 1177.7390.

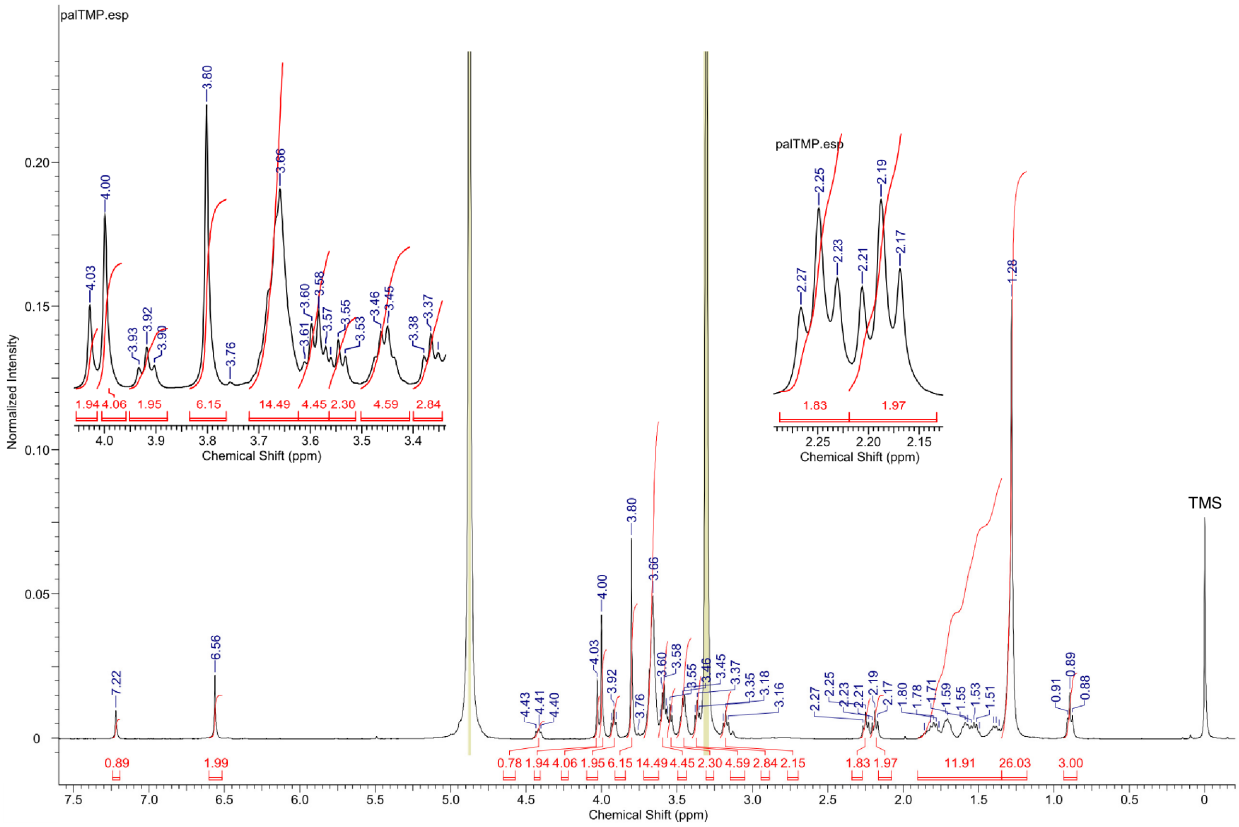
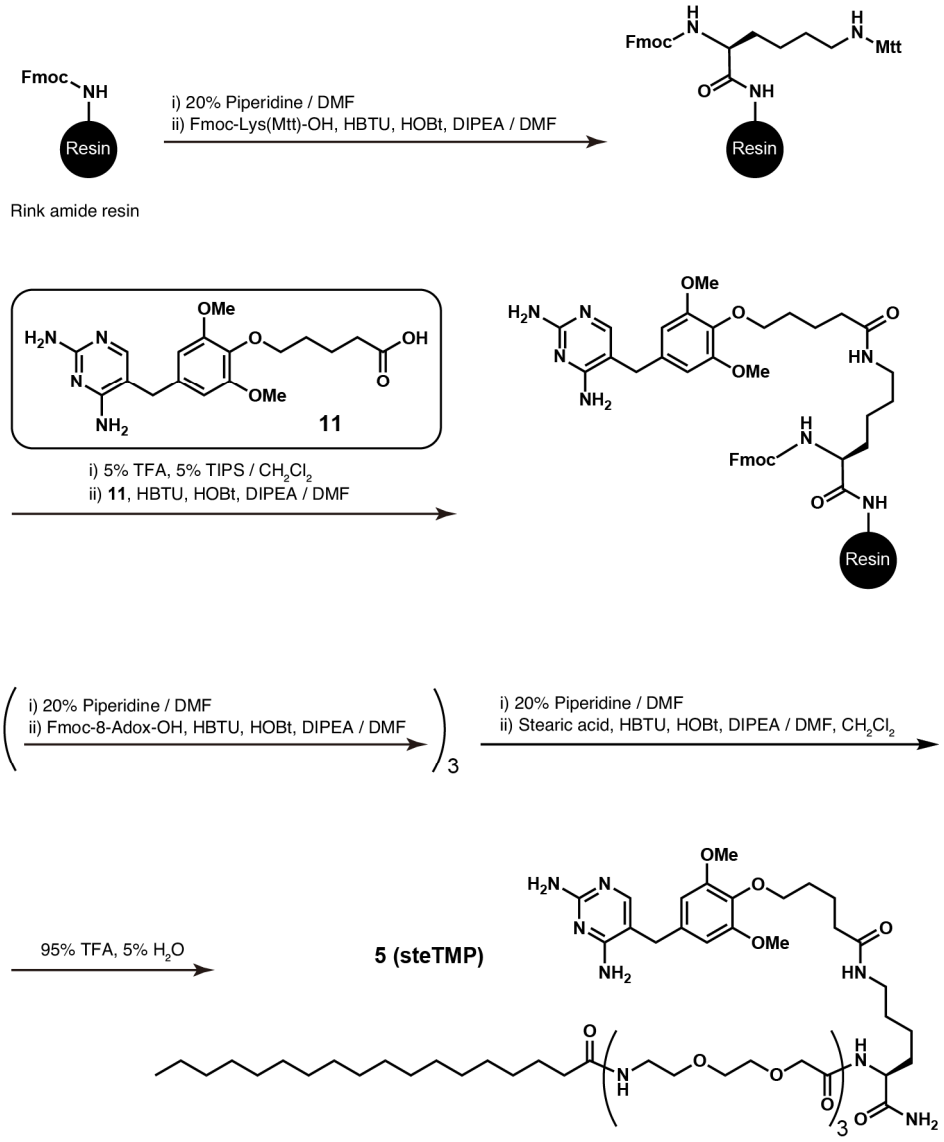


Figure S9. ^{1}H NMR spectrum of **4** (palTMP) in CD_3OD.

Synthesis of Compound 5 (steTMP)



Scheme S5. Synthetic route of **5** (steTMP)

Compound **5** (steTMP) was synthesized on Rink amide resin (0.58 mmol/g) (86 mg, 50 μmol). First, Fmoc-Lys(Mtt)-OH was coupled to the resin. After washing the resin with DMF, MeOH, and CH_2Cl_2, the Mtt group was deprotected by treatment with CH_2Cl_2 containing 5% TFA and 5% TIPS. The resin was then washed with CH_2Cl_2, MeOH, and DMF. **11** was coupled to the side chain of the lysine with a mixture of **11** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. Subsequently, Fmoc deprotection and coupling reactions were repeated using Fmoc-Adox-OH as a building block. The N-terminus was lipidated using a mixture of stearic acid (4.1 eq.), HBTU (4.0 eq.), HOBt

(4.0 eq.), and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing with DMF, MeOH, and CH_2Cl_2, the resin was dried *in vacuo*. Cleavage from the resin was performed with 95% TFA containing 5% H_2O. The crude product was purified by reverse-phase HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **5** (steTMP) as a white solid [21 mg, 35% (as a mono-TFA salt)].

Compound **5** (steTMP)

^{1}H NMR (400 MHz, CD_3OD): δ 7.22 (1H, s), 6.56 (2H, s), 4.41 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.92 (2H, m), 3.80 (6H, s), 3.66 (12H, m), 3.58 (4H, m), 3.55 (2H, m), 3.45 (4H, m), 3.37 (2H, t, J = 6.0 Hz), 3.18 (2H, t, J = 6.0 Hz), 2.25 (2H, t, J = 8.0 Hz), 2.19 (2H, t, J = 8.0 Hz), 1.91–1.35 (12H, m), 1.28 (28H, m), 0.89 (3H, t, J = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1205.7755; found, 1250.7696.

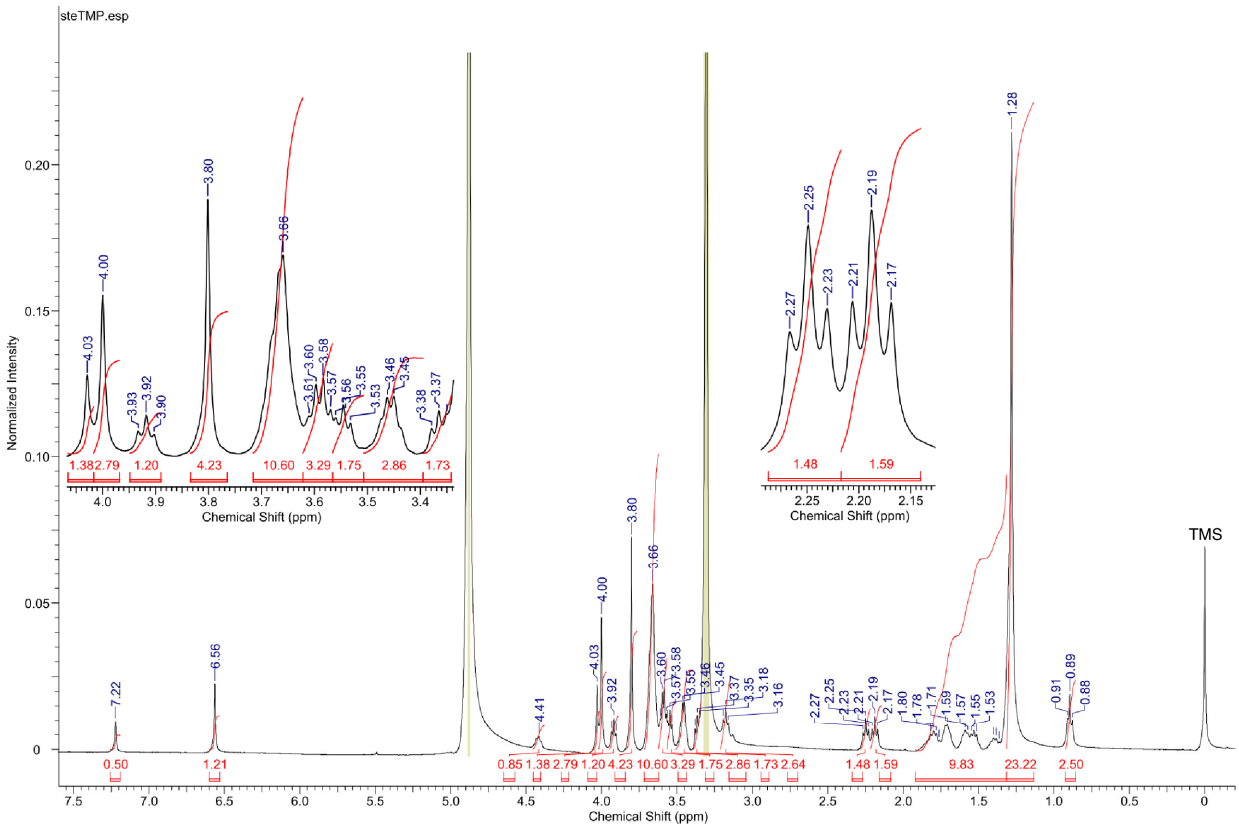
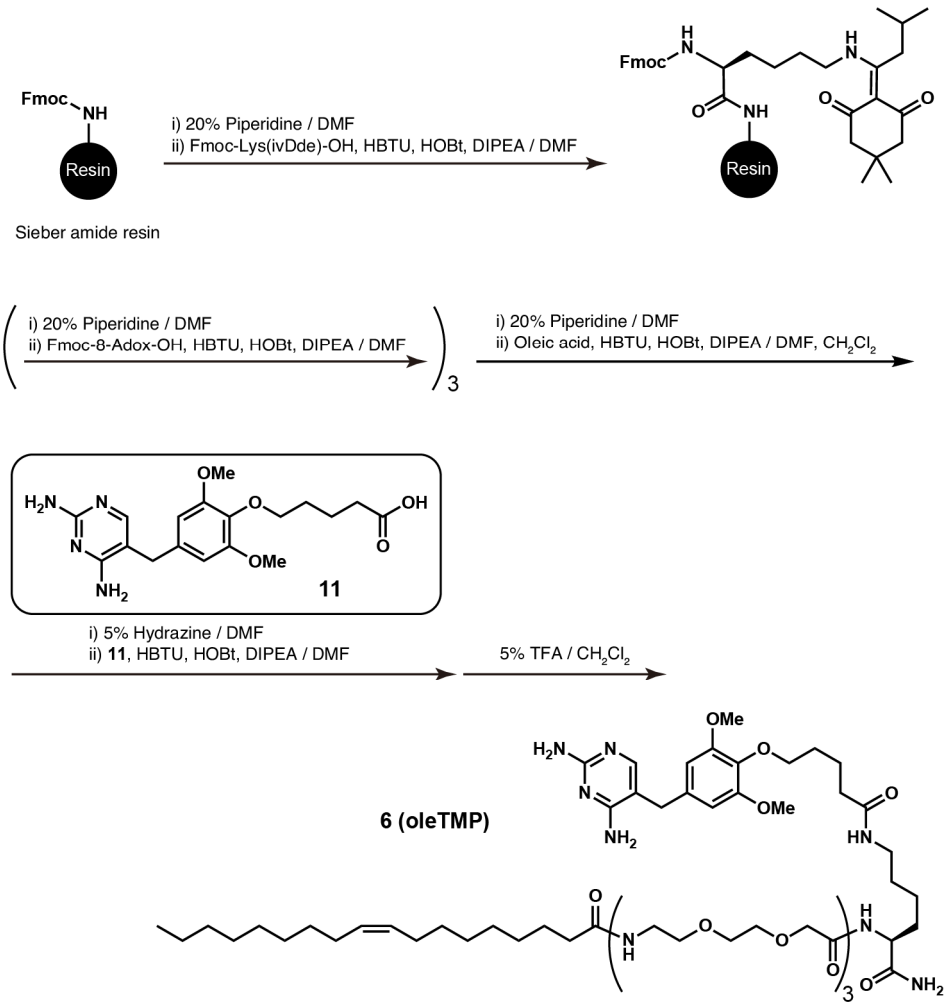


Figure S10. ^{1}H NMR spectrum of **5** (steTMP) in CD_3OD.

Synthesis of Compound 6 (oleTMP)



Scheme S6. Synthetic route of **6** (oleTMP)

Compound **6** (oleTMP) was synthesized on Sieber amide resin (0.55 mmol/g) (73 mg, 40 μmol). First, Fmoc-Lys(ivDde)-OH and Fmoc-Adox-OH (x3) were coupled to the resin. The N-terminus was lipidated using a mixture of oleic acid (4.1 eq.), HBTU (4.0 eq.), HOBt (4.0 eq.), and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing the resin with DMF, the ivDde group was deprotected by treatment with DMF containing 5% hydrazine monohydrate. The resin was then washed with DMF. **11** was coupled to the side chain of the lysine with a mixture of **11** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing with DMF, MeOH, and CH_2Cl_2, the resin was dried *in vacuo*. Cleavage from the resin was performed with CH_2Cl_2 containing 5% TFA. After co-evaporation with toluene, the crude product was purified by reverse-phase HPLC

using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **6** (oleTMP) as a white solid [30 mg, 57% (as a mono-TFA salt)].

Compound **6** (oleTMP)

^{1}H NMR (400 MHz, CD_3OD): δ 7.22 (1H, s), 6.56 (2H, s), 5.34 (2H, m), 4.41 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.92 (2H, m), 3.80 (6H, s), 3.66 (12H, m), 3.58 (4H, m), 3.55 (2H, m), 3.45 (4H, m), 3.37 (2H, t, J = 6.0 Hz), 3.18 (2H, t, J = 6.0 Hz), 2.25 (2H, t, J = 8.0 Hz), 2.19 (2H, t, J = 8.0 Hz), 2.02 (4H, m), 1.91–1.35 (12H, m), 1.35–1.20 (22H, m), 0.90 (3H, t, J = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1203.7599; found, 1203.7545.

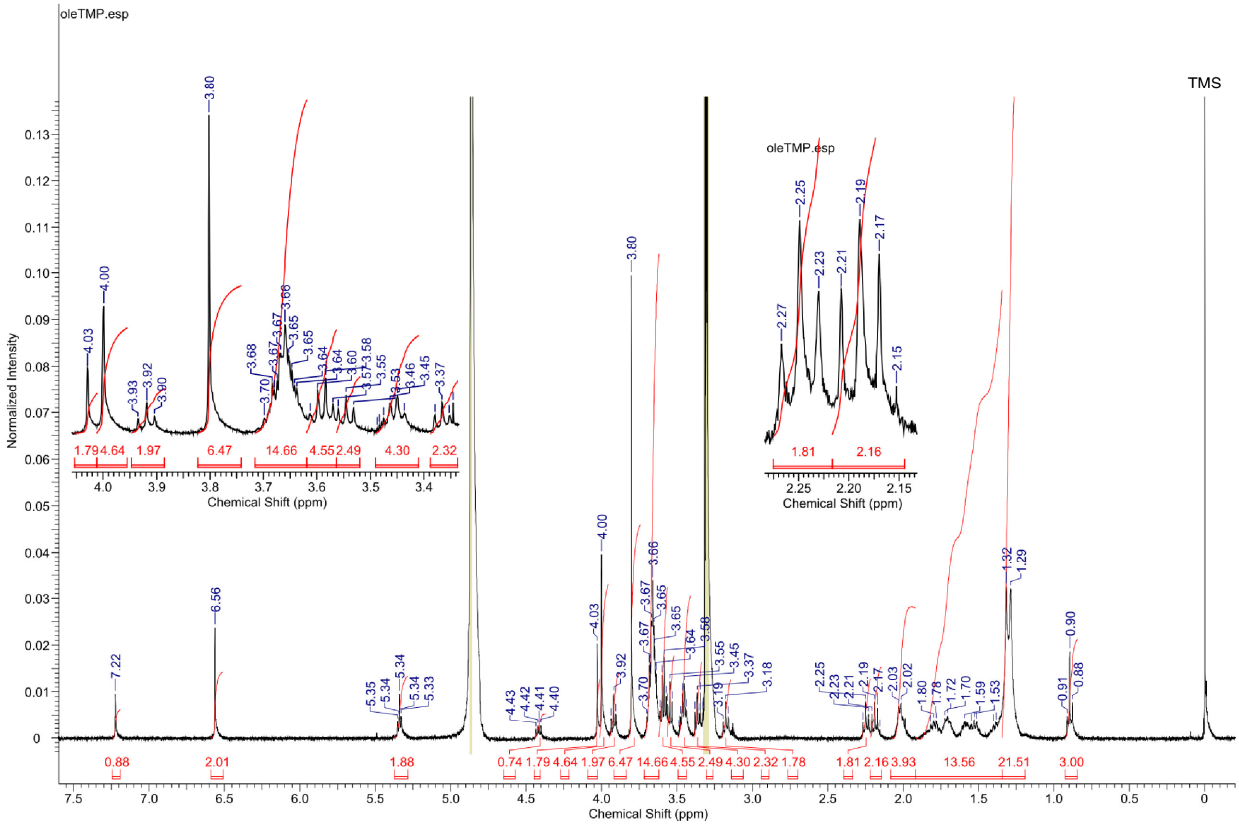
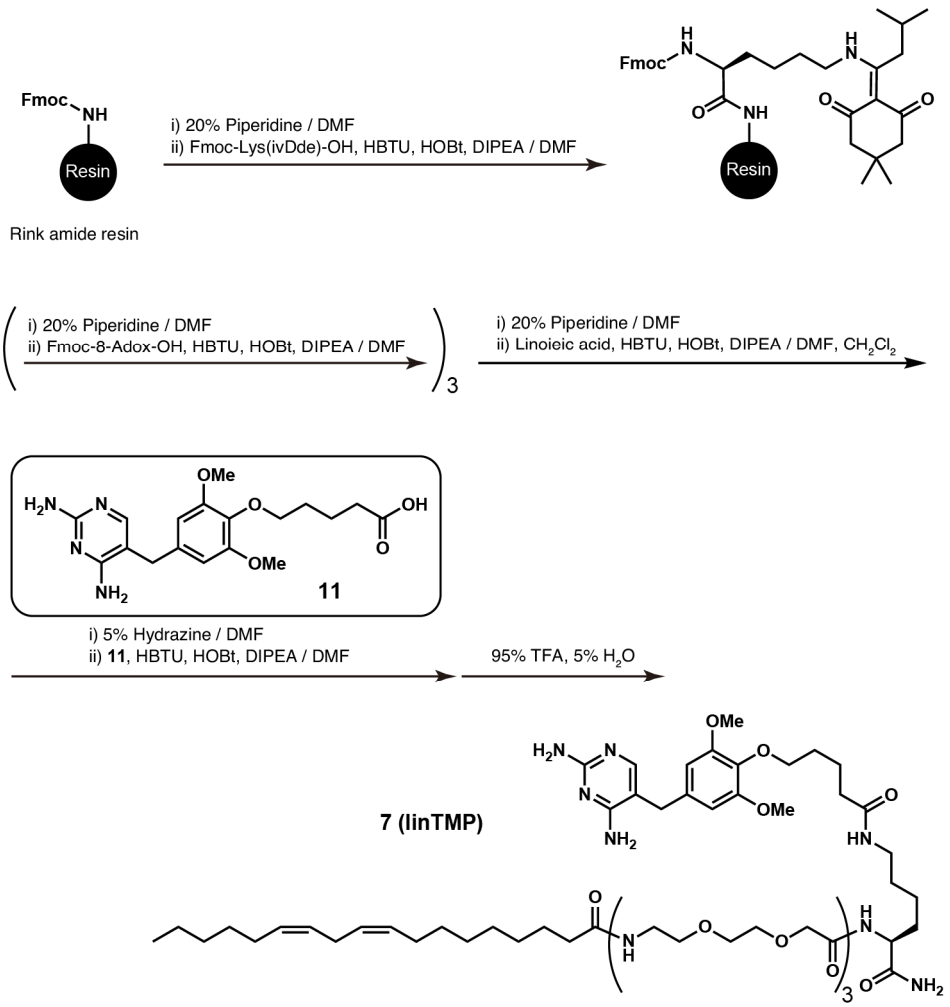


Figure S11. ^{1}H NMR spectrum of **6** (oleTMP) in CD_3OD.

Synthesis of Compound 7 (linTMP)



Scheme S7. Synthetic route of **7** (linTMP)

Compound **7** (linTMP) was synthesized on Rink amide resin (0.58 mmol/g) (35 mg, 20 μmol). First, Fmoc-Lys(ivDde)-OH and Fmoc-Adox-OH (x3) were coupled to the resin. The N-terminus was lipidated using a mixture of linoleic acid (4.1 eq.), HBTU (4.0 eq.), HOBt (4.0 eq.), and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing the resin with DMF, the ivDde group was deprotected by treatment with DMF containing 5% hydrazine monohydrate. The resin was then washed with DMF. **11** was coupled to the side chain of the lysine with a mixture of **11** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing with DMF, MeOH, and CH_2Cl_2, the resin was dried *in vacuo*. Cleavage from the resin was performed with 95% TFA containing 5% H_2O. The crude product was purified by reverse-phase HPLC using a semi-preparative C18 column

(a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **7** (linTMP) as a colorless oil [5.94 mg, 23% (as a mono-TFA salt)].

Compound **7** (linTMP)

^{1}H NMR (400 MHz, CD_3OD): δ 7.25 (1H, s), 6.56 (2H, s), 5.34 (4H, m), 4.41 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.92 (2H, m), 3.80 (6H, s), 3.66 (12H, m), 3.58 (4H, m), 3.55 (2H, m), 3.45 (4H, m), 3.37 (2H, t, J = 6.0 Hz), 3.18 (2H, t, J = 6.0 Hz), 2.77 (2H, m), 2.25 (2H, t, J = 8.0 Hz), 2.19 (2H, t, J = 8.0 Hz), 2.05 (4H, m), 2.01–1.35 (12H, m), 1.35–1.23 (16H, m), 0.90 (3H, t, J = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1201.7442; found, 1201.7388.

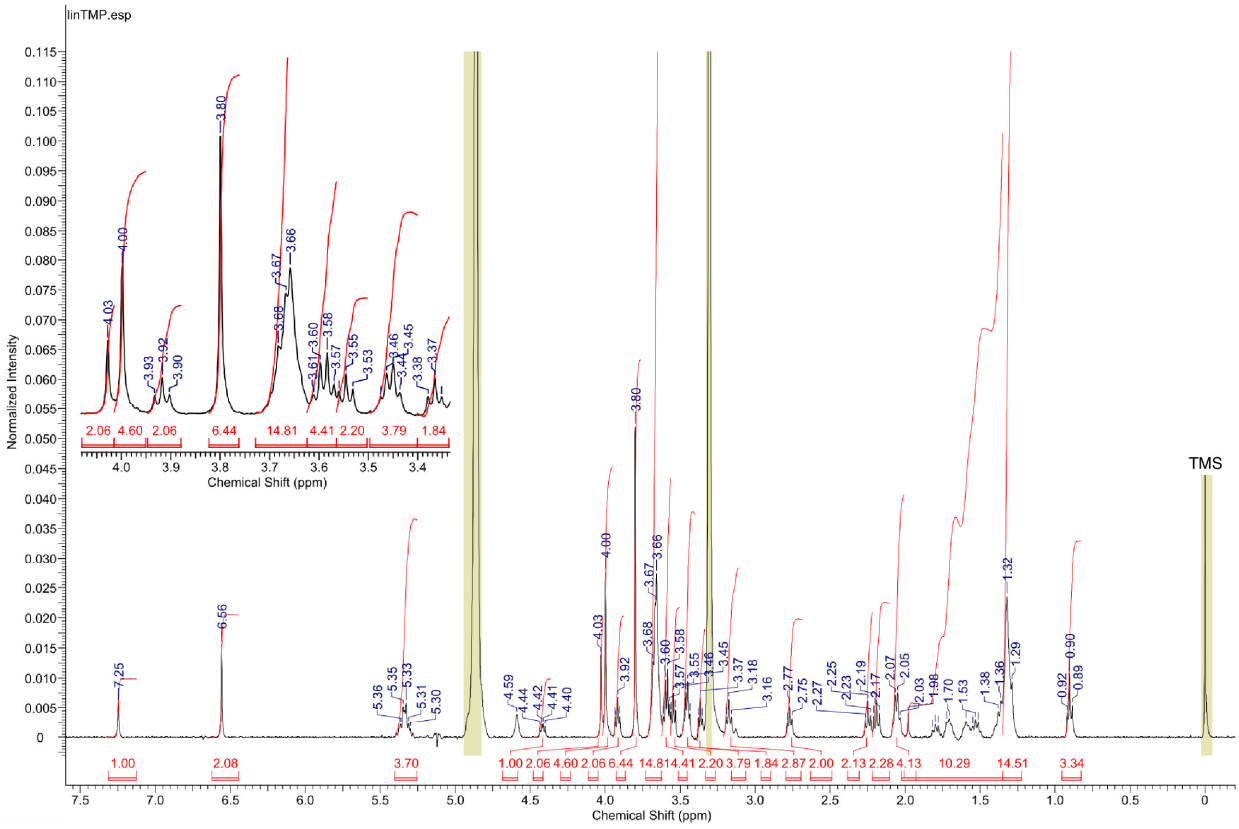
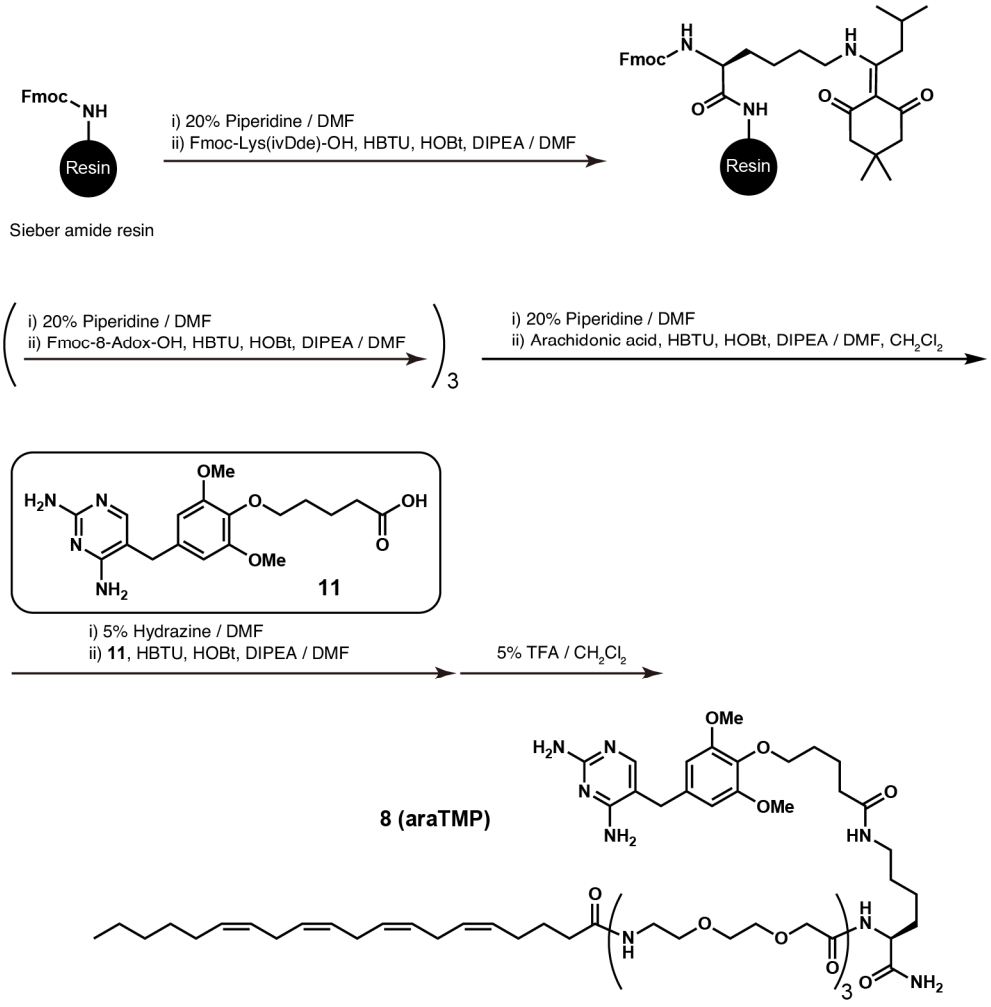


Figure S12. ^{1}H NMR spectrum of **7** (linTMP) in CD_3OD.

Synthesis of Compound 8 (araTMP)



Scheme S8. Synthetic route of **8** (araTMP)

Compound **8** (araTMP) was synthesized on Sieber amide resin (0.69 mmol/g) (29 mg, 20 μmol). First, Fmoc-Lys(ivDde)-OH and Fmoc-Adox-OH (x3) were coupled to the resin. The N-terminus was lipidated using a mixture of arachidonic acid (4.1 eq.), HBTU (4.0 eq.), HOBt (4.0 eq.), and DIPEA (8.0 eq.) in DMF/CH_2Cl_2 (1/1). After washing the resin with DMF, the ivDde group was deprotected by treatment with DMF containing 5% hydrazine monohydrate. The resin was then washed with DMF. **11** was coupled to the side chain of the lysine with a mixture of **11** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. After washing with DMF, MeOH, and CH_2Cl_2, the resin was dried *in vacuo*. Cleavage from the resin was performed with CH_2Cl_2 containing 5% TFA. After co-evaporation with toluene, the crude product was purified by reverse-phase

HPLC using a semi-preparative C18 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **8** (araTMP) as a pale-yellow oil [2.4 mg, 9.5% (as a mono-TFA salt)].

Compound **8** (araTMP)

^{1}H NMR (400 MHz, CD_3OD): δ 7.23 (1H, s), 6.56 (2H, s), 5.35 (8H, m), 4.41 (1H, m), 4.03 (2H, s), 4.00 (4H, s), 3.92 (2H, m), 3.80 (6H, s), 3.66 (12H, m), 3.58 (4H, m), 3.55 (2H, m), 3.45 (4H, m), 3.37 (2H, m), 3.18 (2H, t, J = 6.0 Hz), 2.82 (6H, m), 2.25 (2H, t, J = 8.0 Hz), 2.22 (2H, t, J = 6.0 Hz), 2.08 (4H, m), 2.01–1.35 (12H, m), 1.35–1.10 (8H, m), 0.90 (3H, t, J = 6.0 Hz).

HRMS (ESI): calculated for $[M+H]^+$, 1225.7442; found, 1225.7386.

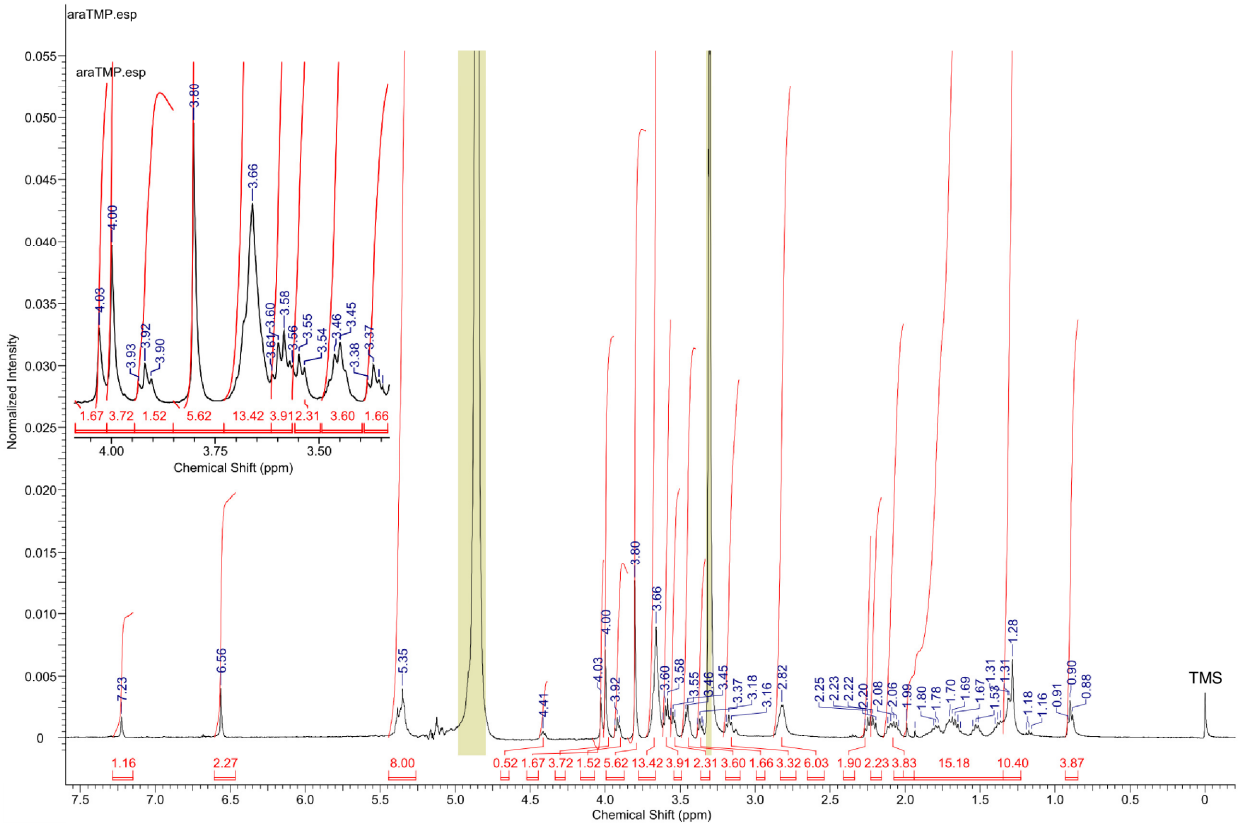
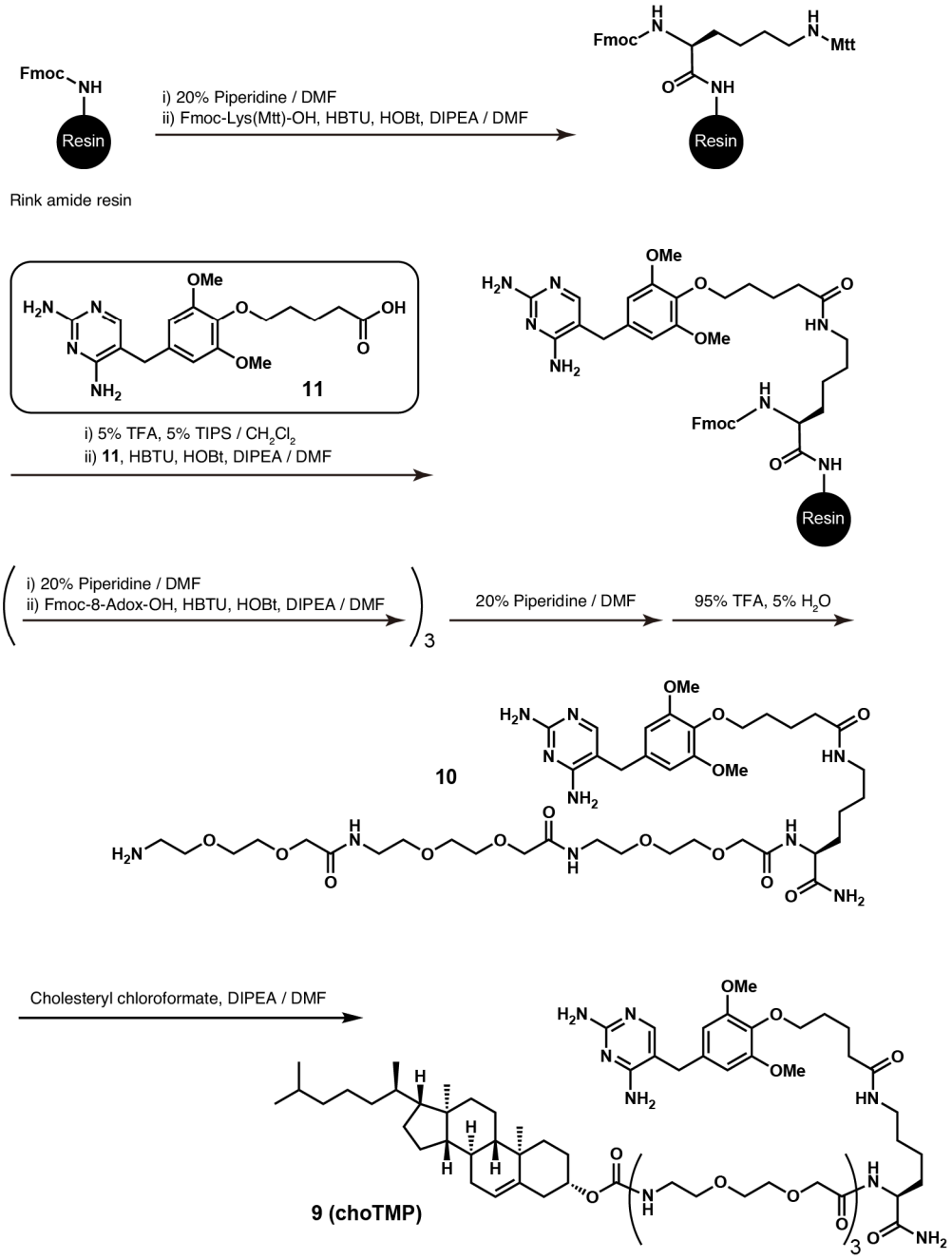


Figure S13. ^{1}H NMR spectrum of **8** (araTMP) in CD_3OD.

Synthesis of Compound 9 (choTMP)



Scheme S9. Synthetic route of **9** (choTMP)

Compound **10** was synthesized on Rink amide resin (0.33 mmol/g) (52 mg, 30 μmol). First, Fmoc-Lys(Mtt)-OH was coupled to the resin. After washing the resin with DMF, MeOH, and CH_2Cl_2, the Mtt group was deprotected by treatment with CH_2Cl_2 containing 5% TFA and 5% TIPS. The resin was then washed with CH_2Cl_2, MeOH, and DMF. **11**

was coupled to the side chain of the lysine with a mixture of **11** (3.1 eq.), HBTU (3.0 eq.), HOBt (3.0 eq.), and DIPEA (6.0 eq.) in DMF. Subsequently, Fmoc deprotection and coupling reactions were repeated using Fmoc-Adox-OH as a building block. After deprotection of the N-terminal Fmoc group, the resin was washed with DMF, MeOH, and CH_2Cl_2 and dried *in vacuo*. Cleavage from the resin was performed with 95% TFA containing 5% H_2O. After evaporation, the crude product **10** was used in the next reaction without further purification.

The crude compound **10** was dissolved in DMF (0.5 mL). Cholesteryl chloride (16 mg, 1.2 eq.) and DIPEA (52 μL, 10 eq.) were added to the solution. After incubation at room temperature for 1 h, the crude product was purified by reverse-phase HPLC using a semi-preparative C4 column (a linear gradient of MeCN containing 0.1% TFA and 0.1% aqueous TFA) to afford **9** (choTMP) as a white solid [19 mg, 43% (as a mono-TFA salt)].

Compound **9** (choTMP)

^{1}H NMR (400 MHz, CD_3OD): δ 7.22 (1H, s), 6.56 (2H, s), 5.38 (1H, m), 4.41 (2H, m), 4.03 (2H, s), 4.00 (4H, s), 3.92 (2H, m), 3.80 (6H, s), 3.66 (12H, m), 3.60 (4H, m), 3.55 (2H, m), 3.45 (6H, m), 3.18 (2H, t, J = 6.0 Hz), 2.96–0.96 (45H, m), 0.93 (3H, d, J = 8.0 Hz), 0.87 (6H, m), 0.71 (3H, s).

HRMS (ESI): calculated for $[M+H]^+$, 1351.8487; found, 1351.8433.

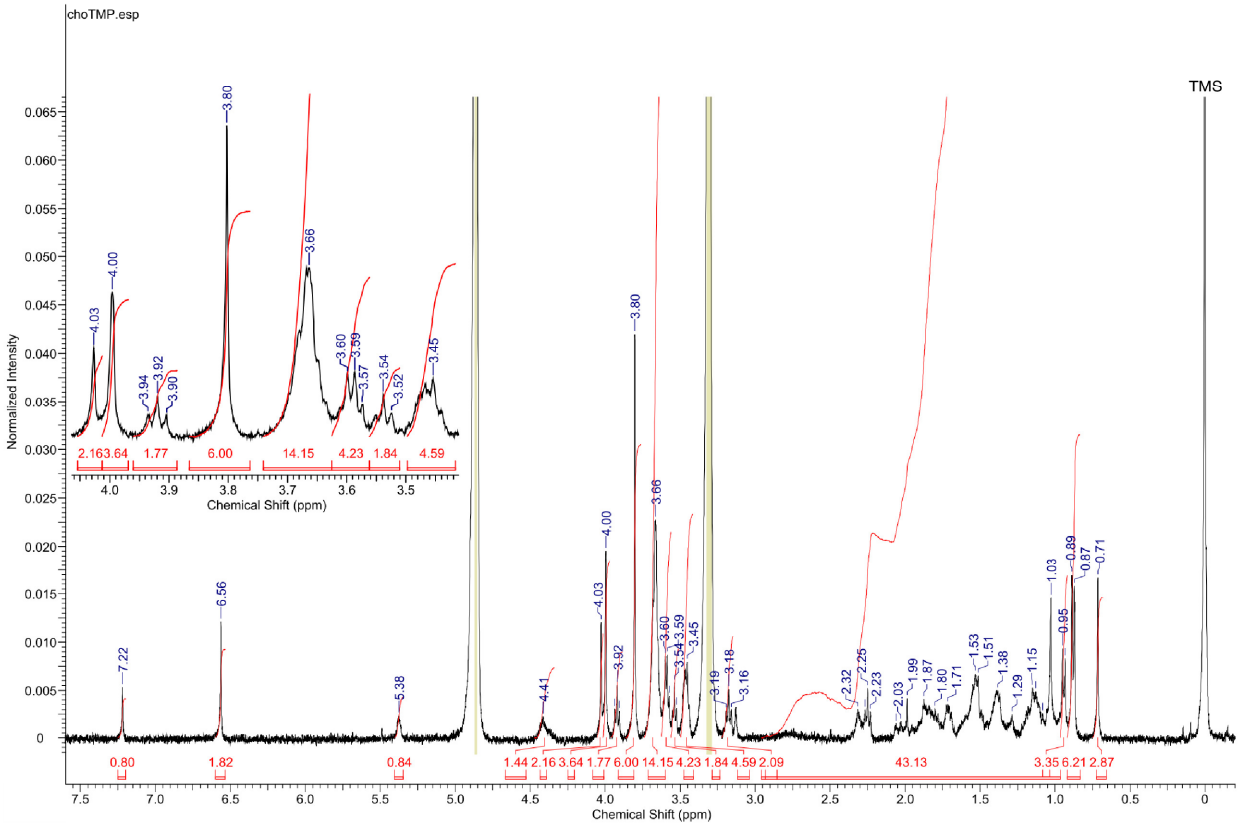


Figure S14. ^{1}H NMR spectrum of **9** (choTMP) in CD_3OD.

Supplementary Methods: Molecular and Cell Biology Experiments

Plasmid construction

All the cDNA and amino acid sequences of the constructs used in this study are listed in **Figures S4** and **S5**. We used pPB-CAG.EBNXN (provided by Dr. Allan Bradley, Wellcome Trust Sanger Institute),[S7] pEGFP-C1 (Clontech), pEGFP-N1 (Clontech), and pmCherry-N1 (Clontech) as vector backbones. We also used pPBbsr[S8] [blasticidin S resistance (*bsr*) gene] and pPBpuro[S9] [puromycin resistance (*pac*) gene] as *piggyBac* donor vectors to establish stable cell lines. We used pcDNA3-HA-H-Ras_wt (Addgene plasmid #39503, provided by Dr. Julian Downward, Francis Crick Institute),[S10] pCX4puro-CRY2-cRaf (provided by Dr. Kazuhiro Aoki, National Institute for Basic Biology),[S11] pCX4neo-mCherry-ERK2-K57R (provided by Dr. Michiyuki Matsuda, Kyoto University),[S4] and pF1KB9098 encoding human RasGRF1 (Kazusa DNA Research Institute) as PCR templates. All expression plasmids were generated using standard cloning procedures.

Cell culture and transfection

HeLa cells were cultured in DMEM (Wako) supplemented with 10% heat-inactivated FBS (Biowest), penicillin (100 U/mL), and streptomycin (100 μg/mL) [DMEM(+)] at 37°C under a humidified 5% CO_2 atmosphere. For transient expression experiments, cells were transfected using Lipofectamine LTX (Invitrogen) or 293fectin (Invitrogen) according to the manufacturer's instructions.

Establishment of stable cell lines

A *piggyBac* transposon system[S7] was used to establish HeLa cell lines stably expressing the indicated construct(s). HeLa cells were co-transfected with a *piggyBac* donor vector(s) (pPBpuro and/or pPBbsr) encoding the desired protein(s) and pCMV-mPBase encoding the *piggyBac* transposase (provided by Dr. Allan Bradley, Wellcome Trust Sanger Institute)[S7,S12] using Polyethylenimine MAX (Polysciences Inc.). Cells were selected with 2 μg/mL puromycin and/or 10 μg/mL blasticidin S for at least 10 days. Bulk populations of selected cells were used.

Live cell imaging

Fluorescence imaging was performed with (i) a LSM880 confocal laser-scanning microscope (Zeiss) equipped with a Plan-Apochromat 63×/1.40 NA oil objective (Zeiss), a Definite Focus.2 module (Zeiss), and an incubation chamber (Incubator PM 2000 RBT, Pecon), or (ii) an IX83/FV3000 confocal laser-scanning microscope (Olympus) equipped with a PlanApo N 60×/1.42 NA oil objective (Olympus), a Z drift compensator system (IX3-ZDC2, Olympus), and a stage top incubator (Tokai Hit). Lasers used for excitation were: 488 nm for EGFP, AcGFP1, and mAG1; 543 nm for mCherry, LysoTracker Red DND99 (Invitrogen), and MitoTracker Red (Invitrogen); 561 nm for mCherry. Unless otherwise noted, time-lapse live cell imaging was performed every 1 or 2 min at 37°C. Fluorescence images were analyzed using the Fiji distribution of ImageJ.[S13]

SLIPT assay

To conduct SLIPT experiments, 2×10^5 HeLa cells stably expressing eDHFR-EGFP[S14] were plated on 35 mm glass-bottomed dishes (Iwaki Glass) and cultured for 24 h at 37°C in 5% CO_2. The medium was changed to serum-free DMEM supplemented with penicillin (100 U/mL) and streptomycin (100 μg/mL) [DMEM(–)], and the cells were observed by time-lapse imaging before and after the addition of the indicated compound (5 or 10 μM) dissolved in DMSO (final DMSO concentration < 0.1% v/v). For palmitoylation inhibition experiments, 2×10^5 HeLa cells expressing eDHFR-EGFP were plated on 35 mm glass-bottomed dishes (Iwaki Glass) and cultured for 24 h at 37°C in 5% CO_2. The cells were incubated with 2-BP (100 μM) in DMEM(+) for 3 h, and the medium was changed to DMEM(–). The cells were then imaged before and after treatment with mgcTMP (5 μM).

Colocalization analysis

For colocalization analysis using ER and Golgi markers, 2×10^5 HeLa cells were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37°C in 5% CO_2. The cells were co-transfected with pCMV-eDHFR-mCherry and pAcGFP-Golgi (Clontech) or pER-mAG1 (Medical & Biological Laboratories) at a 1:1 ratio using Lipofectamine LTX. After incubation for 24 h, the medium was changed to DMEM(–), and the cells were imaged 20 min after treatment with the indicated compound (5 μM). For colocalization analysis using mitochondria and lysosome markers, 2×10^5 HeLa cells expressing eDHFR-EGFP

were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37°C in 5% CO_2. The medium was changed to DMEM(–), and the cells were treated with mgsTMP (5 μM) for 20 min. The cells were stained with MitoTracker Red (0.25 μM) or LysoTracker Red DND99 (0.25 μM) according to the manufacturer's instructions and then imaged.

Artificial activation of endomembrane Ras

HeLa cells (1×10^5) stably expressing $HRas^{C181/184S}$ and RD-RasGEF (or RD) [established using pPBbsr-HA-HRas(C181/184S) and pPBpuro-RD-RasGEF or pPBpuro-RD] were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37°C in 5% CO_2. The cells were transfected with pCMV-EGFP-RBD using 293fectin. After incubation for 24 h, the medium was changed to DMEM(–), and the cells were observed by time-lapse imaging before and after the addition of oleTMP (0.5 μM).

Inhibition of active nuclear transport of ERK by endomembrane trapping

HeLa cells (2×10^5) stably expressing RD-ERK (established using pPBpuro-RD-ERK) were plated on 35 mm glass-bottomed dishes and cultured for 24 h at 37°C in 5% CO_2. The medium was changed to DMEM(–), and the cells were observed by time-lapse imaging before and after the stepwise addition of EGF (Upstate; 50 ng/mL), oleTMP (0.5 μM), and TMP (50 μM).

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