

Rapid one-step biotinylation of biological and non-biological surfaces

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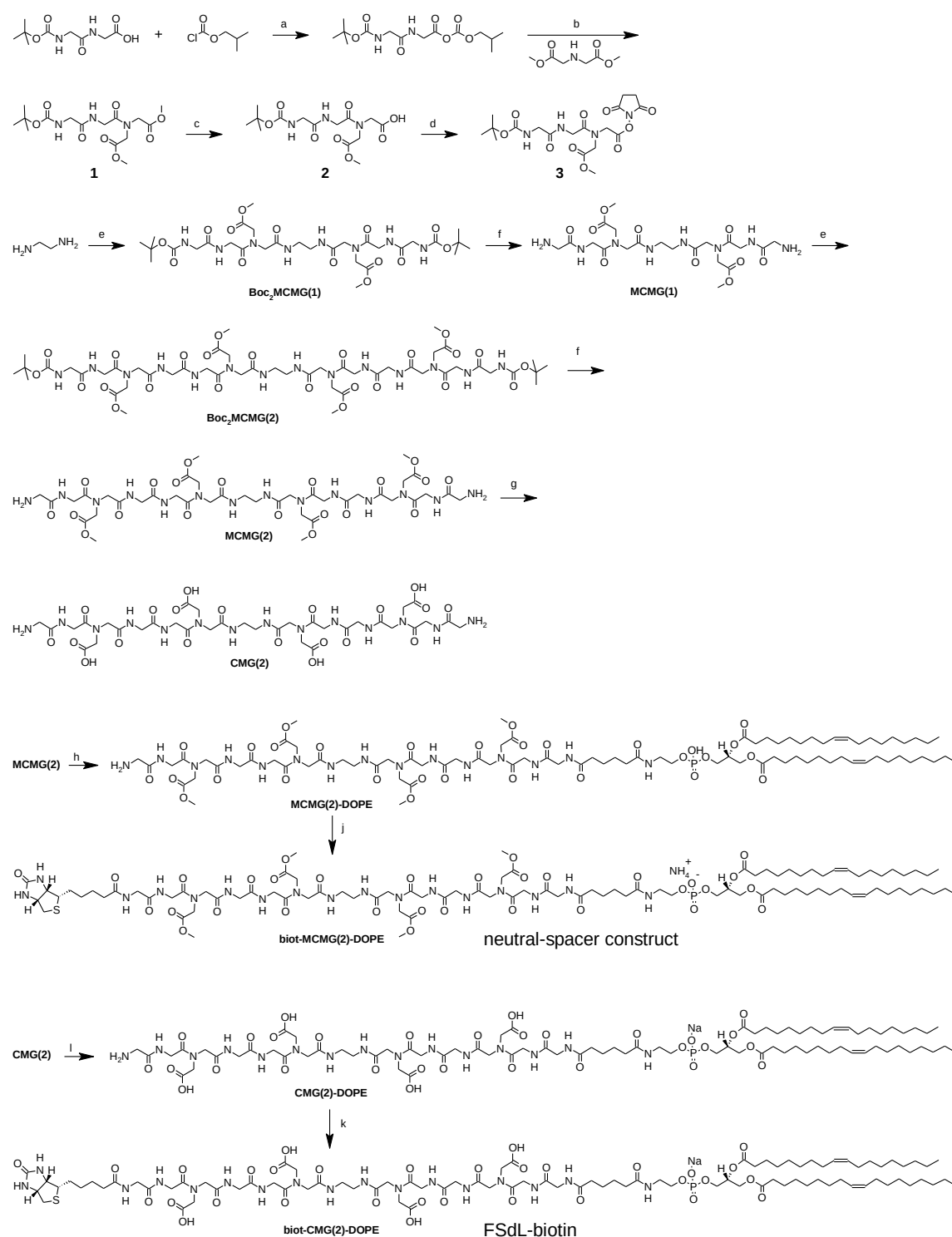
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Supplementary Note 1. **Synthesis.**

Supplementary Note 1a. **Synthetic scheme and detailed protocol for synthesis of FSdL-biotin and corresponding neutral-spacer construct.**

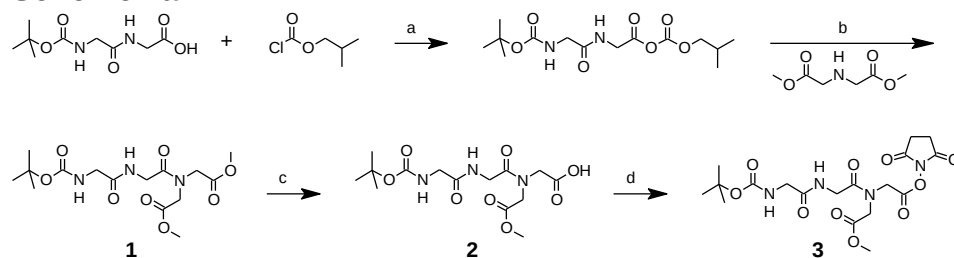


(a) N -methylmorpholine, CH_2Cl_2 , $-15\text{ }^\circ\text{C}$; (b) 1-hydroxybenzotriazole, from $-15\text{ }^\circ\text{C}$ to ambient temperature; (c) NaOH, MeOH/ H_2O ; (d) N,N' -dicyclohexylcarbodiimide, N -hydroxysuccinimide, DMF; (e) **3**, Et_3N , DMSO; (f) CF_3COOH ; (g) Et_3N , H_2O ; (h)

DOPE-Ad-ONSu in dichloroethane, Et₃N, pyridine; (i) DOPE-Ad-ONSu in dichloroethane, aq. NaHCO₃, *i*-PrOH/H₂O; (j) biotin *N*-oxysuccinimide ester, Et₃N, DMF; (k) biotin *N*-oxysuccinimide ester in DMF, aq. NaHCO₃, *i*-PrOH/H₂O.

Preparation of the tripeptide building block 3

Scheme 1a.1



a) *N*-methylmorpholine, CH₂Cl₂, -15 °C; (b) 1-hydroxybenzotriazole, from -15 °C to ambient temperature; (c) NaOH, MeOH/H₂O; (d) *N,N'*-dicyclohexylcarbodiimide, *N*-hydroxysuccinimide, DMF.

Preparation of {[2-(2-*tert*-butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid methyl ester (**1**)

N-Methylmorpholine (11.0 ml, 0.1 mol) was added to a stirred suspension of Boc-glycyl-glycine (23.2 g, 0.1 mol) in 150 ml methylene chloride, the solution was cooled to -15 °C and isobutyl chloroformate (13.7 g, 0.1 mol) was added for 10 min. Then 1-hydroxybenzotriazole and the solution of *N*-(carboxymethyl)glycine dimethyl ester (16.1 g, 0.1 mol) in 50 ml DMF were added to the reaction mixture at the same temperature. The resulting mixture was stirred for 30 min at 0 °C, then for 2 h at ambient temperature and evaporated to dryness. The residue was dissolved in 200 ml of methylene chloride and washed with 100 ml 0.5 M HCl and 200 ml 2% aq. NaHCO₃. Solvents were evaporated in vacuum and the residue was purified with column chromatography on silica gel (3% MeOH in CHCl₃) to give pure target compound (34.08 g, 91%) as a colourless glass.

TLC: R_f = 0.40 (5% MeOH in CHCl₃), R_f = 0.49 (7:1 (v/v) chloroform/methanol).

¹H NMR (500 MHz, [D₆]DMSO, 30 °C) δ, ppm: 7.826 (t, *J*=5.1 Hz, 1H; NHCO), 6.979 (t, *J*=5.9 Hz, 1H; NHCOO), 4.348 and 4.095 (s, 2H; NCH₂COO), 3.969 (d, *J*=5.1 Hz, 2H; COCH₂NH), 3.689 and 3.621 (s, 3H; OCH₃), 3.559 (d, *J*=5.9 Hz, 2H; COCH₂NHCOO), 1.380 (s, 9H; C(CH₃)₃).

Preparation of {[2-(2-tert-butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid (2)

0.2 M aqueous NaOH (325 ml) was added to a stirred solution of **1** (24.42 g, 65.05 mmol) in methanol (325 ml), reaction mixture was kept for 15 min at ambient temperature, acidified with acetic acid (5 ml) and evaporated to dryness. Column chromatography of the residue on silica gel (methanol – ethyl acetate 1:1) gave the target compound as mixture of free acid and Na-salt (20.44 g) which was dissolved in methanol/water/pyridine mixture (20:10:1, 350 ml) and passed through ion-exchange column (Dowex 50X4-400, pyridine form, 300 ml) to remove Na cations. Column was washed with the same mixture, eluate evaporated and dried in vacuum to give pure target compound (20.15 g, 86%) as a white solid.

TLC: R_f = 0.47 (*i*-PrOH / ethyl acetate/water 4:3:1), R_f = 0.34 (MeOH / ethyl acetate 1:1).

^1H NMR (500 MHz, $[\text{D}_6]\text{DMSO}$, 30 °C), mixture of *cis*- and *trans*- conformers of peptide bond formed by *N*-carboxymethylglycine in ~ 3:1 ratio. Major conformer, δ , ppm: 7.717 (t, J =5 Hz, 1H; NHCO), 7.024 (t, J =5.9 Hz, 1H; NHCOO), 4.051 (s, 2H; $\text{NCH}_2\text{COOCH}_3$), 3.928 (d, J =5 Hz, 2H; COCH_2NH), 3.786 (s, 2H; NCH_2COOH), 3.616 (s, 3H; OCH_3), 3.563 (d, J =5.9 Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.381 (s, 9H; $\text{C}(\text{CH}_3)_3$); minor conformer, δ , ppm: 7.766 (t, J =5 Hz, 1H; NHCO), 7.015 (t, J =5.9 Hz, 1H; NHCOO), 4.288 (s, 2H; $\text{NCH}_2\text{COOCH}_3$), 3.928 (d, J =5 Hz, 2H; COCH_2NH), 3.858 (s, 2H; NCH_2COOH), 3.676 (s, 3H; OCH_3), 3.563 (d, J =5.9 Hz, 2H; $\text{COCH}_2\text{NHCOO}$), 1.381 (s, 9H; $\text{C}(\text{CH}_3)_3$).

Preparation of {[2-(2-tert-Butoxycarbonylamino-acetylamino)-acetyl]-methoxycarbonylmethyl-amino}-acetic acid *N*-oxysuccinimide ester (3)

N,N'-Dicyclohexylcarbodiimide (15.83 g, 76.71 mmol) was added to an ice-cooled stirred solution of **2** (26.40 g, 73.06 mmol) and *N*-hydroxysuccinimide (8.83 g, 76.71 mmol) in DMF (210 ml). The mixture was stirred for 30 min at 0 °C then for 2 h at ambient temperature. Precipitated *N,N'*-dicyclohexylurea was filtered off and washed with DMF (80 ml). The filtrate and washings were evaporated in vacuum to a minimal volume, and the residue was stirred with

Et₂O (500 ml) for 1 h. Ether extract was decanted and the residue was dried in vacuum to give target compound as a white foam (32.57 g, 97%).

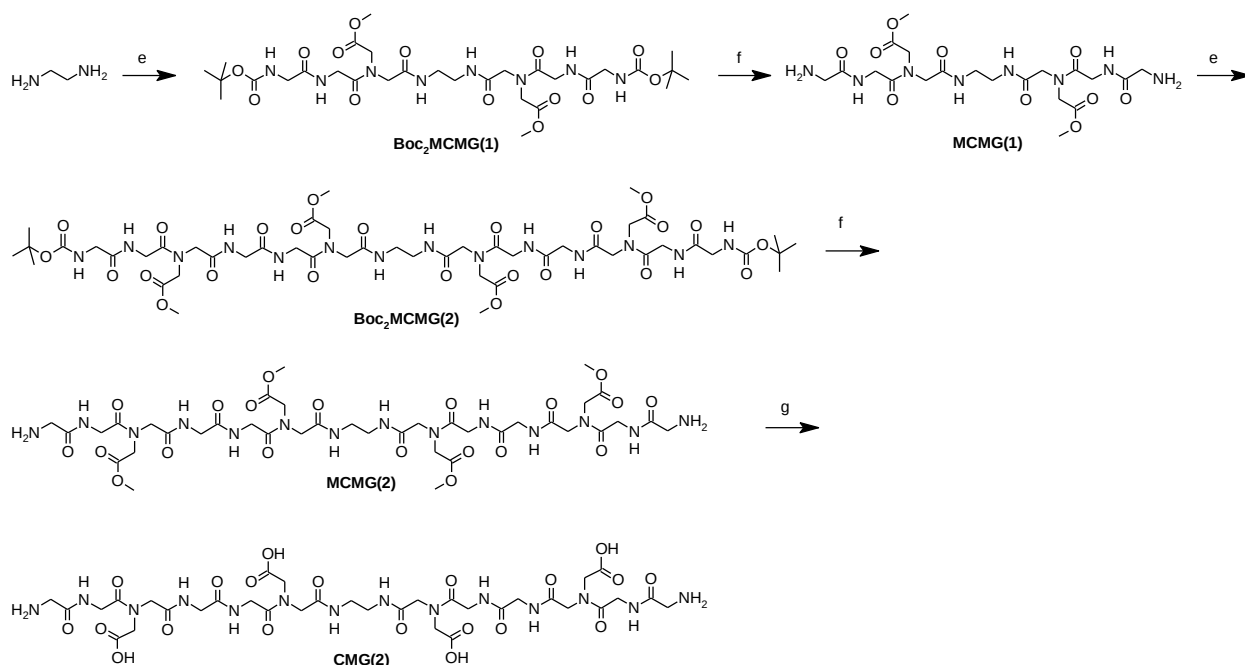
TLC: R_f = 0.71 (acetone/acetic acid 40:1), R_f = 0.43 (ethyl acetate / acetone/ water / acetic acid 50:10:1:1).

¹H NMR (500 MHz, DMSO[D₆], 30 °C), mixture of *cis*- and *trans*- conformers of *N*-carboxymethylglycine unit c. 3:2. Major conformer; δ, ppm: 7.896 (t, *J*=5.1 Hz, 1H; NHCO), 6.972 (t, *J*=5.9 Hz, 1H; NHCOO), 4.533 (s, 2H; NCH₂COON), 4.399 (s, 2H; NCH₂COOCH₃), 3.997 (d, *J*=5.1 Hz, 2H; COCH₂NH), 3.695 (s, 3H; OCH₃), 3.566 (d, *J*=5.9 Hz, 2H; COCH₂NHCOO), 1.380 (s, 9H; C(CH₃)₃).

Minor conformer; δ, ppm: 7.882 (t, *J*=5.1 Hz, 1H; NHCO), 6.963 (t, *J*=5.9 Hz, 1H; NHCOO), 4.924 (s, 2H; NCH₂COON), 4.133 (s, 2H; NCH₂COOCH₃), 4.034 (d, *J*=5.1 Hz, 2H; COCH₂NH), 3.632 (s, 3H; OCH₃), 3.572 (d, *J*=5.9 Hz, 2H; COCH₂NHCOO), 1.380 (s, 9H; C(CH₃)₃).

Synthesis of MCMG(2) and CMG(2) diamines

Scheme 1a.2



(e) **3**, Et₃N, DMSO; (f) CF₃COOH; (g) Et₃N, H₂O.

Preparation of Boc₂MCMG(1)

A solution of ethylenediamine (808 mg, 13.44 mmol) and Et₃N (1.87 ml, 13.5 mmol) in DMSO (5 ml) was added to a stirred solution of **3** (15.42 g, 33.64 mmol) in DMSO (50 ml). The reaction mixture was stirred for 30 min at ambient temperature and acidified with acetic acid (1.2 ml), then fractionated with Sephadex LH-20 column (column volume 1200 ml, eluent – MeOH/water 2:1 + 0.2% AcOH). Fractions containing Boc₂MCMG(1) were combined, solvents evaporated and the residue was concentrated in vacuum. The product was additionally purified by silica gel column chromatography using 2-propanol/ethyl acetate/water (2:6:1) as eluent. Fractions containing pure Boc₂MCMG(1) were combined, solvents evaporated and a residue was dried in vacuum to give target Boc₂MCMG(1) as colourless foam (8.41 g, 84 %).

TLC: R_f = 0.48 (*i*-PrOH/ ethyl acetate/water 2:3:1).

¹H NMR (500 MHz, [D₆]DMSO, 30°C), mixture of conformers ~3:2: 8.166, 8.125, 7.917 and 7.895 (m, total 2H; 2 CONHCH₂), 7.793 (m, 2H; NHCH₂CH₂NH), 7.001 (br. t, 2H; 2 NHCOO), 4.277-3.893 (total 12H; 2 CH₂COO, 4 NCH₂CO), 3.690 and 3.635 (s, total 6H; 2 COOCH₃), 3.567 (d, *J*=5.8 Hz, 4H; 2 CH₂NHCOO), 3.131 (m, 4H; NHCH₂CH₂NH), 1.379 (s, 18H; 2 C(CH₃)₃) ppm.

MS (MALDI-TOF): calc. for C₃₀H₅₀N₈O₁₄ M_w_{isotopic}=746, found 769 [M+Na], 785 [M+K].

Preparation of MCMG(1)

Trifluoroacetic acid (25 ml) was added to a stirred solution of Boc₂MCMG(1) (4.88 g, 6.535 mmol) in methylene chloride (25 ml) and the solution was kept for 1 h at ambient temperature. Then a reaction mixture was concentrated and the residue was evaporated three times with anhydrous MeOH (50 ml), then a residue was extracted three times with Et₂O (100 ml) to remove traces of trifluoroacetic acid. The resulted precipitate (as a white solid) was dried to give 5.06 g (~100 %) of MCMG(1) as bis-trifluoroacetic salt.

TLC: R_f = 0.23 (ethanol/water/pyridine/acetic acid 5:1:1:1).

^1H NMR (500 MHz, D_2O , 30°C), mixture of conformers (~5:4): 4.400-4.098 (total 12H; 2 CH_2COO , 4 NCH_2CO), 3.917 (s, 4H; 2 COCH_2NH_2), 3.829 and 3.781 (s, total 6H; 2 COOCH_3), 3.394 (m, 4H; $\text{NHCH}_2\text{CH}_2\text{NH}$) ppm.
MS (MALDI-TOF): calc. for $\text{C}_{20}\text{H}_{34}\text{N}_8\text{O}_{10}$ $\text{MW}_{\text{isotopic}}=546$, found 547 [M+H], 569 [M+Na], 585 [M+K].

Preparation of Boc₂MCMG(2)

A solution of **3** (7.49 g, 16.34 mmol) in DMSO (17 ml) and Et_3N (2.73 ml, 19.6 mmol) was added to the stirred solution of MCMG(1) bis-trifluoroacetic salt (5.06 g, 6.535 mmol) in DMSO (13 ml). The reaction mixture after stirring for 2 h at ambient temperature was acidified with acetic acid (4.0 ml) and fractionated with Sephadex LH-20 column chromatography (column volume 1200 ml, eluent – MeOH/water 2:1 + 0.2% AcOH). Fractions containing pure Boc₂MCMG(2) were combined, solvents evaporated and the residue was dried in vacuum to give target Boc₂MCMG(2) as colourless foam (7.82 g, 97 %).

TLC: $R_f=0.25$ (iPrOH/ ethyl acetate/water 2:3:1).

^1H NMR (500 MHz, $[\text{D}_6]\text{DMSO}$, 30°C), mixture of conformers: 8.393-7.887 (total 6H; 6 CONHCH_2), 7.775 (m, 2H; $\text{NHCH}_2\text{CH}_2\text{NH}$), 6.996 (br. t, 2H; 2 NHCOO), 4.299-3.730 (total 28H; 4 CH_2COO , 10 NCH_2CO), 3.691 and 3.633 (s, total 12H; 4 COOCH_3), 3.564 (d, $J=5.8$ Hz, 4H; 2 CH_2NHCOO), 3.129 (m, 4H; $\text{NHCH}_2\text{CH}_2\text{NH}$), 1.380 (s, 18H; 2 $\text{C}(\text{CH}_3)_3$) ppm.
MS (MALDI-TOF): calc. for $\text{C}_{48}\text{H}_{76}\text{N}_{14}\text{O}_{24}$ $\text{MW}_{\text{isotopic}}=1233$, found 1256 [M+Na], 1272 [M+K].

Preparation of MCMG(2) diamine

Boc₂MCMG(2) (1212 mg, 0.983 mmol) was dissolved in CF_3COOH (4 ml) and the solution was kept for 30 min at r.t. Trifluoroacetic acid was evaporated in vacuum and the residue was extracted three times with Et_2O (trituration with 25 ml of Et_2O followed by filtration) to remove residual CF_3COOH , and the obtained white powder was dried in vacuum. Yield of MCMG(2) bis-trifluoroacetic salt was 1238 mg (~100 %). The powder was dissolved in 8 mL of water and then was freeze-dried. Weight of freeze-dried MCMG(2) bis-

trifluoroacetic salt was larger than theoretical by 10% due to stability of hydrates.

TLC: $R_f = 0.21$ (ethanol/water/pyridine/acetic acid 5:1:1:1).

^1H NMR (500 MHz, $[\text{D}_2]\text{H}_2\text{O}$, 30°C), mixture of conformers: 4.430-4.014 (total 28H; 4 CH_2COO , 10 NCH_2CO), 3.911 (s, 4H; 2 COCH_2NH_2), 3.823 and 3.772 (s, total 12H; 4 COOCH_3), 3.386 (m, 4H; $\text{NHCH}_2\text{CH}_2\text{NH}$) ppm.

MS (MALDI-TOF): calc. for $\text{C}_{38}\text{H}_{60}\text{N}_{14}\text{O}_{20}$ $\text{Mw}_{\text{isotopic}}=1032$, found 1033 $[\text{M}+\text{H}]$, 1055 $[\text{M}+\text{Na}]$.

Preparation of CMG(2) diamine

To the solution of MCMG(2) bis-trifluoroacetic salt (618 mg, 0.49 mmol) in water (20 mL) Et_3N (0.5 mL, 3.6 mmol) was added, and the solution was kept for 15 h at r.t.. The reaction mixture was evaporated to dryness, the residue dissolved in water (3 mL) and the solution was desalted on Sephadex LH-20 column (column volume 250 mL, eluent - MeOH/water 1:1 + 0.05 M pyridine acetate). Fractions, containing pure CMG(2), were evaporated to ~4 mL volume and freeze dried. Yield of CMG(2) diamine (internal salt) was 431 mg (90%).

For large-scale syntheses CMG(2) diamine may be used without desalting on Sephadex, freeze-drying of the residue after evaporation of the reaction mixture gave di-TFA, di- Et_3N salt with some excess of Et_3N (0.35 M/M by NMR data) in quantitative yield.

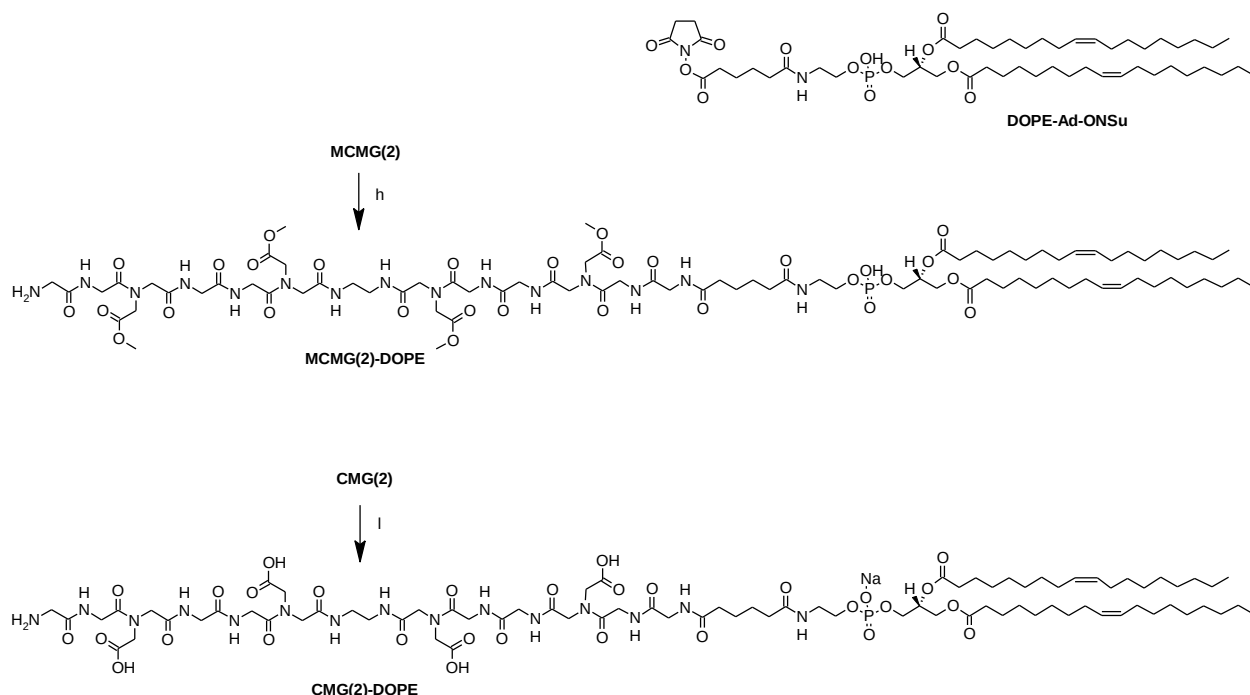
TLC: $R_f = 0.50$ ($^i\text{PrOH}$ /MeOH/acetonitrile/water 4:3:3:4 + 3% conc. aq. NH_3), $R_f = 0.43$ (MeOH/ CHCl_3 /water 2:1:1), $R_f = 0.19$ (MeOH/ CHCl_3 /water 3:1:1).

^1H NMR of CMG(2) internal salt (500 MHz, $[\text{D}_2]\text{H}_2\text{O}$, 30°C), mixture of conformers: 4.328-4.006 (total 28H; 4 CH_2COO , 10 NCH_2CO), 3.907 (s, 4H; 2 COCH_2NH_2), 3.381 (m, 4H; $\text{NHCH}_2\text{CH}_2\text{NH}$) ppm.

MS (MALDI-TOF): calc. for $\text{C}_{34}\text{H}_{52}\text{N}_{14}\text{O}_{20}$ $\text{Mw}_{\text{isotopic}}=976$, found 977 $[\text{M}+\text{H}]$, 999 $[\text{M}+\text{Na}]$, 1015 $[\text{M}+\text{K}]$.

Synthesis of MCMG(2) and CMG(2) DOPE derivatives

Scheme 1a.3



(h) DOPE-Ad-ONSu in dichloroethane, Et₃N, pyridine; (i) DOPE-Ad-ONSu in dichloroethane, aq. NaHCO₃, *i*-PrOH/H₂O.

Preparation of DOPE-Ad-ONSu¹

To a solution of *bis*(N-hydroxysuccinimidyl) adipate (700 mg, 2.05 mmol) in dry *N,N*-dimethyl formamide (30 ml) were added 1,2-O-dioleoyl-*sn*-glycero-3-phosphatidylethanolamine (DOPE) (300 mg, 0.403 mmol) in chloroform (15 ml) and triethylamine (50 μ l). The mixture was stirred for 2 h at room temperature, then neutralized with acetic acid and concentrated in *vacuo*. Column chromatography on Sephadex LH-20 (chloroform/methanol 1:1, 0.2% acetic acid) of the residue yielded the activated lipid (370 mg, 95%) as a colorless syrup.

TLC: R_f = 0.50 (chloroform/methanol/water 12:6:1).

¹H NMR (500 MHz, [D₃]CHCl₃/[D₄]CH₃OH 1:1, 30°C): 5.50 (m, 4H, 2×(-CH=CH-), 5.39 (m, 1H, -OCH₂-CH=O-CH₂O-), 4.58 (dd, 1H, J=3.67, J=11.98, -CCOOHCH-CHO-CH₂O-), 4.34 (dd, 1H, J=6.61, J=11.98, -CCOOHCH-CHO-CH₂O-), 4.26 (m, 2H, PO-CH₂-CH₂-NH₂), 4.18 (m, 2H, -CH₂-OP), 3.62 (m, 2H, PO-CH₂-CH₂-NH₂), 3.00 (s, 4H, ONSuc), 2.80 (m, 2H, -CH₂-CO (Ad), 2.50 (m,

4H, 2×(-CH₂-CO), 2.42 (m, 2H, -CH₂-CO (Ad)), 2.17 (m, 8H, 2×(-CH₂-CH=CH-CH₂-), 1.93 (m, 4H, COCH₂CH₂CH₂CH₂CO), 1.78 (m, 4H, 2×(COCH₂CH₂-), 1.43, 1.47 (2 bs, 40H, 20 CH₂), 1.04 (~t, J≈7 Hz, 6H; 2 CH₃)

Preparation of MCMG(2)-Ad-DOPE amine¹

To the intensively stirred solution of MCMG(2) (hydrated, 10% of H₂O, 378 mg, 0.270 mmol) in pyridine (5 mL) a solution of DOPE-Ad-ONSu (174 mg, 0.180 mmol) in dichloroethane (0.87 mL) and Et₃N (0.1 mL, 0.72 mmol) were added. The reaction mixture was stirred for 2 h and then acidified with 0.2 mL of AcOH and evaporated to minimal volume at 35°C. The residue was dried in vacuum (solid foam). The obtained mixture was separated on silica gel column (2.8 x 33 cm, ~ 200 mL of silica gel in CHCl₃/MeOH 5:1). The mixture was placed on column in CHCl₃/MeOH (5:1) and the components were eluted with CHCl₃/MeOH (1:1) and then with alteration of MeOH/CHCl₃/water composition from 7:7:1 to 6:6:1. First eluted was DOPE-Ad-MCMG(2)-Ad-DOPE (R_f = 0.72, MeOH/CHCl₃/water 6:6:1), second eluted was aimed MCMG(2)-Ad-DOPE amine (R_f = 0.34, MeOH/CHCl₃/water 6:6:1). Remaining MCMG(2) diamine was eluted with MeOH/1 M Py·HOAc (3:2, R_f = 0.47), recovery was 129 mg (42%) as MCMG(2) diacetate. Fractions, containing pure MCMG(2)-Ad-DOPE amine were combined, evaporated and dried in vacuum. The residue was dissolved in 5% AcOH and freeze-dried. Yield of MCMG(2)-Ad-DOPE amine was 132 mg (26% on loaded or 45% on used up MCMG(2)).

¹H NMR (500 MHz, [D₃]CHCl₃/[D₄]CH₃OH 1:2, 30°C): 5.507 (m, 4H; 2 CH₂CH=CHCH₂), 5.406 (m, 1H; OCH₂CHCH₂O), 4.605 (dd, J_{gem}=12.1 Hz, 1H; OCHCHCH₂O), 4.510-3.849 (total 51H; 4 CH₂COO, 12 NCH₂CO, OCHCHCH₂O, OCH₂CH₂N, 4CH₂COOCH₃), 3.586 (m, 4H; NHCH₂CH₂NH), 2.491 and 2.419 (m, total 8H; 4 CH₂CO), 2.194 (m, 8H; 2 CH₂CH=CHCH₂), 1.837 and 1.784 (m, total 8H; 4 CH₂CH₂CO), 1.478 (m, 40H; 20 CH₂), 1.061 (~t, J≈7 Hz, 6H; 2 CH₃) ppm.

MS (MALDI-TOF): calc. for C₈₅H₁₄₄N₁₅O₃₀P M_w_{isotopic}=1886, found 1887 [M+H], 1909 [M+Na], 1925 [M+K].

Preparation of CMG(2)-Ad-DOPE amine¹

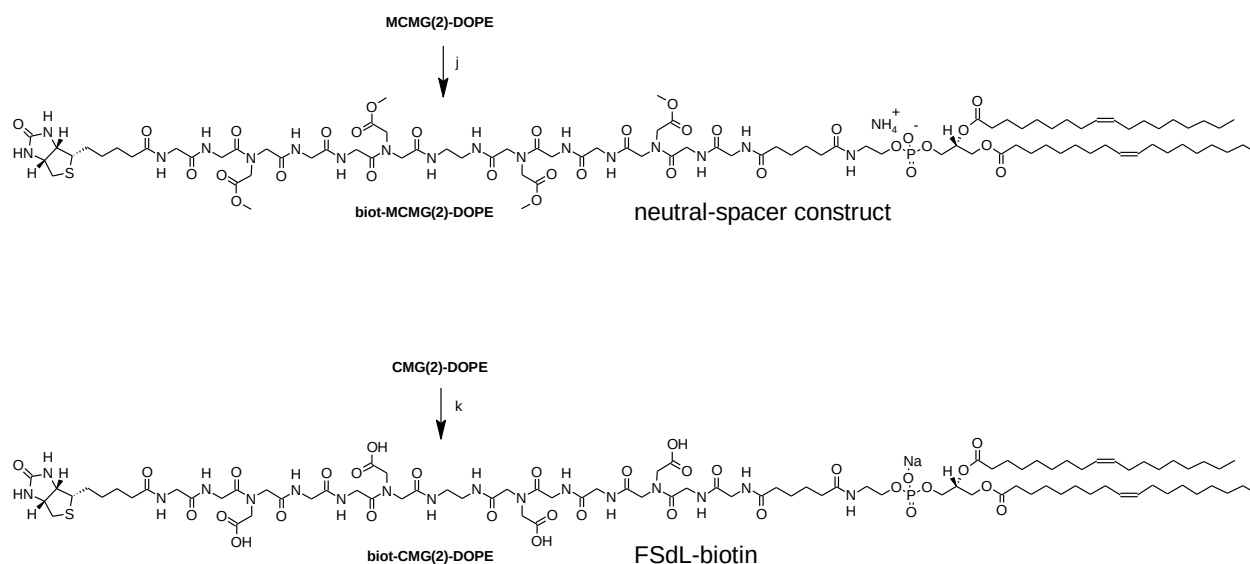
To the intensively stirred solution of CMG(2) diamine (425 mg, 0.435 mmol of internal salt) in *i*-PrOH/water mixture (*i*-PrOH/water 3:2, 10 mL) a solution of DOPE-Ad-ONSu (211 mg, 0.218 mmol) in dichloroethane (0.4 mL) and 1 M aq. solution of NaHCO₃ (0.435 mL, 0.435 mmol) were added. The reaction mixture was stirred for 2 h and then acidified with 0.2 mL of AcOH and evaporated to minimal volume at 35°C. The solid residue was dried in vacuum (solid foam) and then thoroughly extracted with CHCl₃/MeOH mixture (CHCl₃/MeOH 4:1, several times with 10 mL, TLC control). The extracted residue consisted of unreacted CMG(2) and salts (~ 50% of CMG(2) was recovered by desalting of combined the residue and a fractions after chromatography on silica gel according to procedure described in the CMG(2) synthesis.). The combined CHCl₃/MeOH extracts (solution of CMG(2)-Ad-DOPE amine, DOPE-Ad-CMG(2)-Ad-DOPE, N-oxysuccinimide and some CMG(2)) were evaporated in vacuum and dried. The obtained mixture was separated on silica gel column (2.8 x 33 cm, ~ 200 mL of silica gel in CHCl₃/MeOH 5:1). The mixture was placed on column in MeOH/CHCl₃/water mixture (MeOH/CHCl₃/water 6:3:1 + 0.5% of pyridine) and the components were eluted in a stepwise ternary gradient: MeOH/CHCl₃/water composition from 6:3:1 to 6:2:1 and then to 6:2:2 (all with 0.5% of pyridine). DOPE-Ad-CMG(2)-Ad-DOPE was eluted first ($R_f = 0.75$, MeOH/CHCl₃/water 3:1:1), followed by desired DOPE-Ad-CMG(2)amine ($R_f = 0.63$, MeOH/CHCl₃/water 3:1:1), last eluted was CMG(2) ($R_f = 0.31$, MeOH/CHCl₃/water 3:1:1). Fractions, containing pure CMG(2)-Ad-DOPE amine were combined and evaporated to dryness. To remove any low molecular weight impurities and solubilised silica gel the residue was dissolved in ⁱPrOH/water 1:2 mixture (2 mL), and was passed through Sephadex LH-20 column (column volume 130 mL, eluent - ⁱPrOH/water 1:2 + 0.25% of pyridine). Fractions containing pure CMG(2)-Ad-DOPE amine were combined and evaporated (~ 20% of 2-propanol was added to prevent foaming) to dryness, the residue was dissolved in water (~4 mL) and freeze-dried. Yield of CMG(2)-Ad-DOPE amine was 270 mg (68% on DOPE-Ad-ONSu, 34% on loaded or 68% on used up CMG(2) diamine).

^1H NMR (500 MHz, $[\text{D}_2]\text{H}_2\text{O}/[\text{D}_4]\text{CH}_3\text{OH}$ 2:1, 30°C): 5.505 (m, 4H; $2\text{CH}_2\text{CH}=\text{CHCH}_2$), 5.476 (m, 1H; $\text{OCH}_2\text{CHCH}_2\text{O}$), 4.626 (dd, $J_{\text{gem}}=11.6$ Hz, 1H; $\text{OCHCHCH}_2\text{O}$), 4.461-4.084 (total 37H; 4 CH_2COO , 11 NCH_2CO , $\text{OCHCHCH}_2\text{O}$, $\text{OCH}_2\text{CH}_2\text{N}$), 4.002 (s, 2H; COCH_2NH_2), 3.573 (m, 4H; $\text{NHCH}_2\text{CH}_2\text{NH}$), 2.536-2.463 (m, total 8H; 4 CH_2CO), 2.197 (m, 8H; $2\text{CH}_2\text{CH}=\text{CHCH}_2$), 1.807 (m, 8H; 4 $\text{CH}_2\text{CH}_2\text{CO}$), 1.480 (m, 40H; 20 CH_2), 1.063 (~t, $J\approx 6$ Hz, 6H; 2 CH_3) ppm.

MS (MALDI-TOF): calc. for $\text{C}_{81}\text{H}_{136}\text{N}_{15}\text{O}_{30}\text{P}$ $M_{\text{w isotopic}}=1830$, found 1831 $[\text{M}+\text{H}]$, 1853 $[\text{M}+\text{Na}]$, 1869 $[\text{M}+\text{K}]$.

Synthesis of biotin-MCMG(2) and biotin-CMG(2) DOPE derivatives

Scheme 1a.4



(j) biotin *N*-oxysuccinimide ester, Et_3N , DMF; (k) biotin *N*-oxysuccinimide ester in DMF, aq. NaHCO_3 , *i*-PrOH/ H_2O .

Preparation of biot-MCMG(2)-DOPE (neutral-spacer construct)

To the stirred solution of MCMG(2)-Ad-DOPE amine (9.7 mg, 5.14 micromole) in DMF (0.6 mL) the solution of biotin *N*-oxysuccinimide ester (2.3 mg, 6.74 micromole) in DMF (50 μL) and Et_3N (1.7 μL , 12.2 micromole) were added. The reaction mixture was stirred for 3 h at r.t. and then acidified with AcOH (20 μL). The resulted solution was passed through Sephadex LH-20 column (80 mL, eluent – *i*-PrOH/water 1:2 + 0.5% of pyridine and 0.25% of AcOH).

Fractions, containing pure biotin-MCMG(2)-Ad-DOPE were evaporated and dried in vacuum. The residue was dissolved i-PrOH/water (1:4 by volume), aqueous 1 M NH₃ was added (10 µL) and the solution was freeze-dried. Yield of biotin-MCMG(2)-Ad-DOPE (ammonium salt) was 10.3 mg (94%).

TLC: R_f = 0.57 (CHCl₃/methanol/water 6:6:1 by volume).

¹H NMR (700 MHz, [D₄]CH₃OH/[D₂]H₂O/CDCl₃ 4:1:1, 30°C) δ 5.523 (m, 4H; 2 CH₂CH=CHCH₂), 5.430 (m, 1H; OCH₂CHCH₂O), 4.717 (dd, J = 7.6, 5.1 Hz, 1H; NHCH of biotin), 4.627 (dd, J = 12.1, 2.7 Hz, 1H; OCHCHCH₂O), 4.566-3.854 (m, total 52H; NHCH of biotin, 4 CH₂COO, 12 NCH₂CO, OCHCHCH₂O, OCH₂CH₂N, 4 OCH₃), 3.553 (m, 4H; NHCH₂CH₂NH), 3.412 (m, 1H; NHCHCH of biotin), 3.13 (dd, J = 12.8, 5.1 Hz, 1H; NHCHCH of biotin), 2.919 (d, J = 12.8 Hz, 1H; NHCHCH of biotin), 2.548-2.423 (m, total 10H; 5 CH₂CO), 2.205 (m, 8H; 2 CH₂CH=CHCH₂), 1.960-1.750 (m, 12H; CH₂CH₂CH₂CH₂CO of biotin, 5 CH₂CH₂CO), 1.651 (m, 2H; CH₂CH₂CH₂CH₂CO), 1.504 (m, 40H; 20 CH₂), 1.068 (t, J = 7.0 Hz, 6H; 2 CH₃).

MS (MALDI-TOF): calc. for C₉₅H₁₅₈N₁₇O₃₂PS MW_{isotopic}=2112, found 2113 [M+H], 2135 [M+Na], 2151 [M+K].

Insoluble in water, soluble in water/2-propanol mixtures.

Preparation of biot-CMG(2)-DOPE (FSdL-biotin)¹

To the intensively stirred solution of CMG(2)-Ad-DOPE amine (366 mg, 0.200 mmol) in i-PrOH/water mixture (i-PrOH/water 1:1, 15 mL) 1 M aq. solution of NaHCO₃ (1.2 mL, 1.2 mmol) and then the solution of biotin N-oxysuccinimide ester (75.1 mg, 0.220 mmol) in DMF (1.5 mL) were added. The reaction mixture was stirred for 2.5 h and then acidified with 0.15 mL of AcOH and evaporated to minimal volume (beginning of a precipitate formation) at 35°C. The residue was diluted with water (1.5 mL) and 0.05 mL of pyridine was added. The resulted solution was passed through Sephadex LH-20 column (column volume 300 mL, eluent - i-PrOH/water 1:2 + 0.5% of pyridine and 0.25% of AcOH). Fractions, containing pure biotin-CMG(2)-Ad-DOPE were combined and evaporated (~ 20% of 2-propanol was added to prevent foaming) to dryness, the residue was dissolved in water (~ 4 mL, containing 0.05 mL of pyridine) and freeze-dried. Yield of biotin-CMG(2)-Ad-DOPE (pyridinium salt)

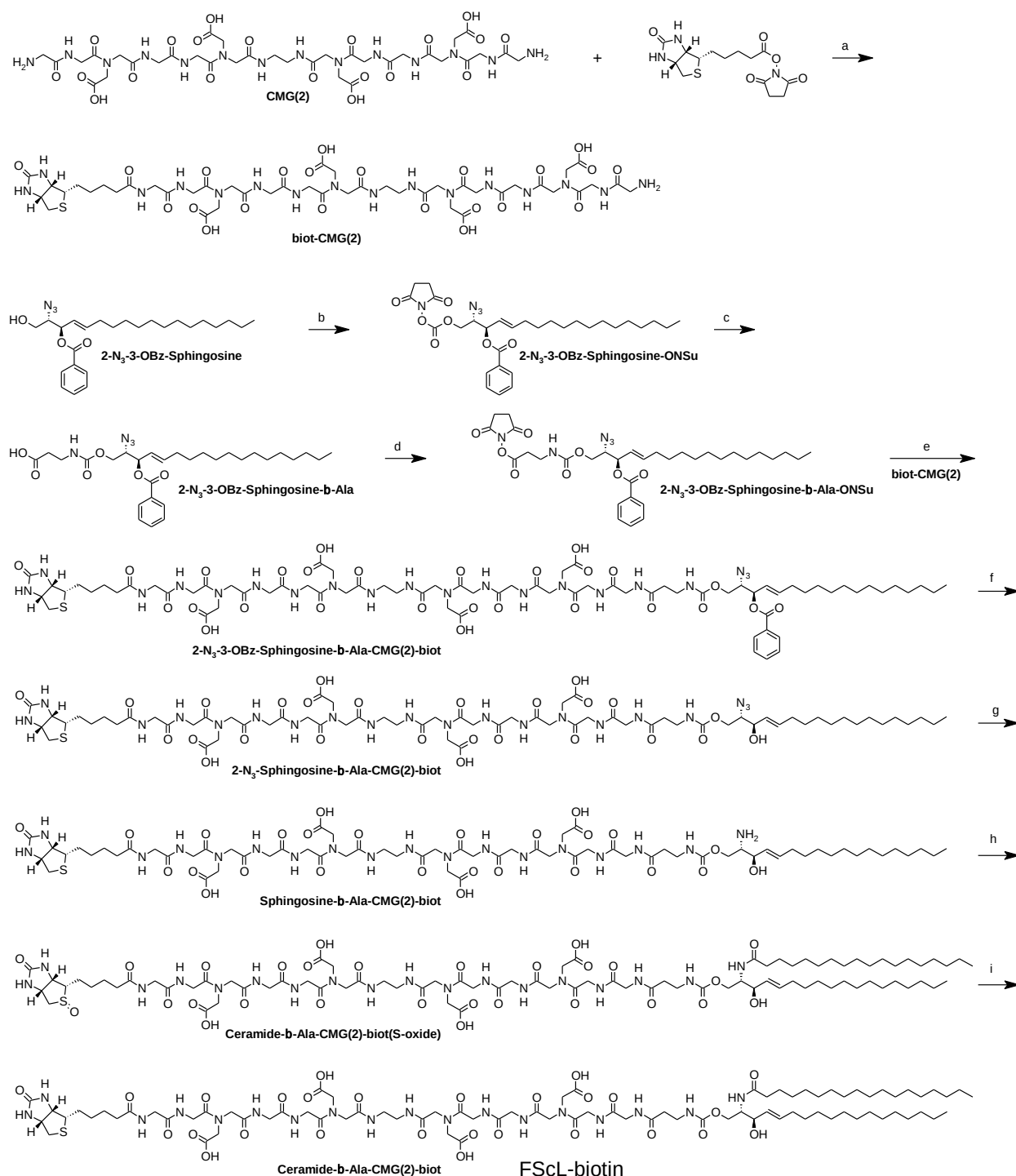
was 384.5 mg (~0.180 mmol). The product was dissolved in water (3 ml) containing NaHCO₃ (0.18 ml of 1 M aqueous solution, 0.180 mmol) and freeze-dried. Yield of biotin-CMG(2)-Ad-DOPE (sodium salt) was 374.3 mg (91% on CMG(2)-Ad-DOPE amine).

TLC: R_f = 0.64 (MeOH/CHCl₃/water 6:2:1).

¹H NMR (600 MHz, [D₂]H₂O/[D₄]CH₃OH 2:1, 30°C): 5.508 (m, 4H; 2 CH₂CH=CHCH₂), 5.458 (m, 1H; OCH₂CHCH₂O), 4.766 (dd, J=7.9 Hz, J=4.9 Hz, 1H; NHCH of biotin), 4.619 (dd, J_{gem}=11.6 Hz, 1H; OCHCHCH₂O), 4.584 (dd, J=7.9 Hz, J=4.5 Hz, 1H; NHCH of biotin), 4.470-4.078 (total 39H; 4 CH₂COO, 12 NCH₂CO, OCHCHCH₂O, OCH₂CH₂N), 3.571 (m, 4H; NHCH₂CH₂NH), 3.483 (m, 1H; NHCHCH of biotin), 3.162 (dd, J=13 Hz, J=4.9 Hz, 1H; NHCHCH of biotin), 2.941 (dd, J=13 Hz, J<2 Hz, 1H; NHCHCH of biotin), 2.527-2.458 (m, total 10H; 5 CH₂CO), 2.196 (m, 8H; 2 CH₂CH=CHCH₂), 1.885 (m, 2H; CH₂CH₂CH₂CH₂CO of biotin), 1.806 (m, 10H; 5 CH₂CH₂CO), 1.627 (m, 2H; CH₂CH₂CH₂CH₂CO), 1.480 (m, 40H; 20 CH₂), 1.069 (~t, J≈7 Hz, 6H; 2 CH₃) ppm.

MS (MALDI-TOF): calc. for C₉₁H₁₅₀N₁₇O₃₂PS Mw_{isotopic}=2056, found 2079 [M+Na], 2095 [M+K], 2101 [MNa+Na].

Supplementary Note 1b. Synthetic scheme and detailed protocol for synthesis of FScL-biotin.

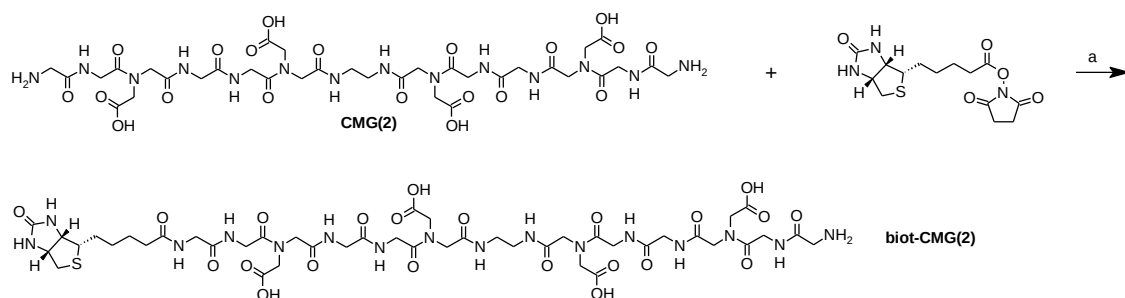


(a) biotin *N*-oxysuccinimide ester in DMF, aq. NaHCO₃, *i*-PrOH/H₂O; (b) disuccinimidylcarbonate, Et₃N, DMF/CH₂Cl₂ (c) β-alanine, Et₃N, DMSO/DMF/CH₂Cl₂; (d) disuccinimidylcarbonate, Et₃N, DMF/1,2-dichloroethane; (e) biot-CMG(2) amine, aq. NaHCO₃, 1,2-dichloroethane/DMSO/H₂O; (f) Et₃N, H₂O/MeOH; (g) dithiothreitol,

Et₃N, H₂O/MeOH; (h) Stearoyl-ONSu in 1,2-dichloroethane, aq. NaHCO₃, H₂O/2-propanol; (i) N-Methyl-mercaptoacetamide. H₂O, 69 h at 40 °C.

Preparation of biotin-CMG(2) amine

Scheme 1b.1



(a) biotin *N*-oxysuccinimide ester in DMF, aq. NaHCO₃, *i*-PrOH/H₂O.

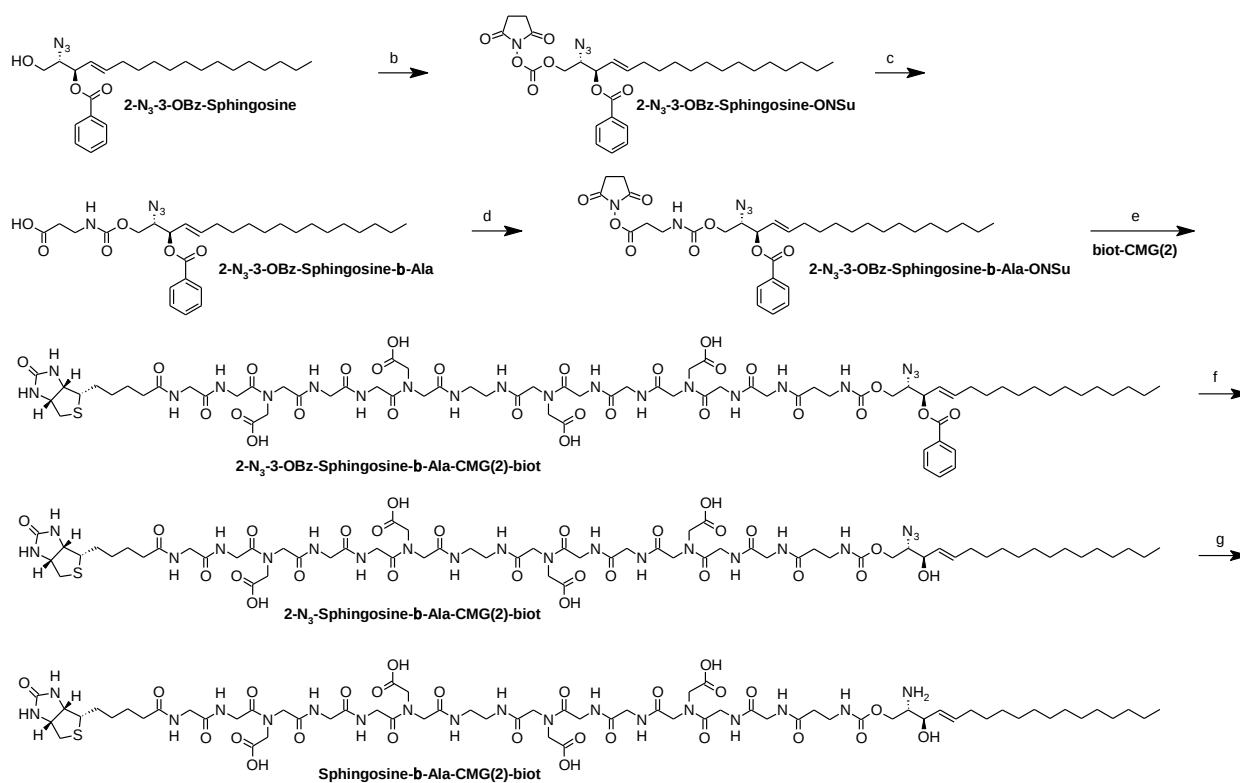
Preparation of biot-CMG(2) amine

To a thoroughly stirred solution of CMG(2) diamine internal salt (693 mg, 0.709 mM; described in Supplementary Note 1) in a mixture of 2-propanol/water (1:1 by volume, 20 mL) a solution of biot-ONSu (242 mg, 0.709 mM) in DMF (4.84 mL) and 1 M aqueous NaHCO₃ (2.84 mL) were added, and the mixture was stirred for 30 min at ambient temperature. The reaction mixture was acidified with AcOH (122 µL), evaporated and dried. The obtained mixture was separated on silica gel column (~ 350 mL of silica gel in CHCl₃/MeOH 4:1). The mixture was placed on column in MeOH/CHCl₃/water mixture (1:4:3 by volume, 4 mL) and the components were eluted with MeOH/CHCl₃/water (1:3:1 by volume). Di-biotin derivative biot-CMG(2)-biot was eluted first (TLC: R_f = 0.48, MeOH/CHCl₃/water 3:1:1), followed by desired biot-CMG(2) amine (TLC: R_f = 0.30, MeOH/CHCl₃/water 3:1:1), last eluted was CMG(2) diamine (TLC: R_f = 0.19, MeOH/CHCl₃/water 3:1:1). Fractions, containing pure biot-CMG(2) amine were combined and evaporated to dryness. To remove any low molecular weight impurities and solubilised silica gel the residue was dissolved in water (2 mL), and was passed through Sephadex LH-20 column (column volume 170 mL, eluent - MeOH/water 1:3 by volume). After evaporating a residue was dissolved in water (~4 mL) and freeze-dried. Yield of biot-CMG(2) amine tri-Na salt was 316 mg (35% on loaded or 51% on used up CMG(2) diamine). Yield of biot-CMG(2)-biot was 190 mg (18% on CMG(2) diamine) and recovery of CMG(2) diamine was 231 mg (32%).

^1H NMR of biot-CMG(2) amine (700 MHz, $[\text{D}_2]\text{H}_2\text{O}$, 30°C): δ 4.637 (dd, $J = 7.9$, 4.6 Hz, 1H; NHCH_2 of biotin), 4.458 (dd, $J = 7.9$, 4.5 Hz, 1H; NHCH_2 of biotin), 4.321-3.900 (total 32H; 4 CH_2COO , 12 NCH_2CO), 3.438-3.337 (m, 5H; $\text{NCH}_2\text{CH}_2\text{N}$ and NHCHCH_2 of biotin), 3.030 (dd, $J = 13.0$, 5.0 Hz, 1H; NHCHCH_2 of biotin), 2.812 (d, $J = 13.0$ Hz, 1H; NHCHCH_2 of biotin), 2.384 (~t, $J = 7.4$ Hz, 2H; COCH_2 of biotin), 1.761, (m, 1H; $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ of biotin), 1.697 (m, 2H; $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ of biotin), 1.621 (m, 1H; $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ of biotin), 1.467 (m, 2H; $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ of biotin) ppm.
 MS (MALDI-TOF): calc. for $\text{C}_{44}\text{H}_{66}\text{N}_{16}\text{O}_{22}\text{S}$ $\text{MW}_{\text{isotopic}}=1202$, found 1203 $[\text{M}+\text{H}]$, 1225 $[\text{M}+\text{Na}]$.

Preparation of sphingosine- β -Ala-CMG(2)-biot

Scheme 1b.2



(b) disuccinimidylcarbonate, Et_3N , DMF/ CH_2Cl_2 (c) β -alanine, Et_3N , DMSO/DMF/ CH_2Cl_2 ; (d) disuccinimidylcarbonate, Et_3N , DMF/1,2-dichloroethane; (e) biot-CMG(2) amine, aq. NaHCO_3 , 1,2-dichloroethane/DMSO/ H_2O ; (f) Et_3N , $\text{H}_2\text{O}/\text{MeOH}$; (g) dithiothreitol, Et_3N , $\text{H}_2\text{O}/\text{MeOH}$.

2-Azido-3-benzoyl-sphingosine-ONSu

To a stirred solution of (2S,3R,4E)-2-azido-3-benzoyloxy-octadec-4-ene-1-ol (2-azido-3-benzoyl-sphingosine²) (103 mg, 0.240 mmol) in a mixture of CH₂Cl₂ (3 mL) and dimethyl formamide (2 mL) disuccinimidylcarbonate (123 mg, 0.480 mmol) and Et₃N (33 μ L, 0.24 mmol) were added, and the mixture was stirred for 20 h at ambient temperature. The reaction mixture was evaporated in vacuum (oil pump), the residue was dissolved in CHCl₃ and extracted with water (3 x 4 mL). Chloroform extract was evaporated and the residue was thoroughly dried in vacuum. Yield of 2-azido-3-benzoyl-sphingosine-ONSu was 130 mg (95%), white solid.

TLC: 2-azido-3-benzoyl-sphingosine R_f = 0.42, 2-Azido-3-benzoyl-Sphingosine-ONSu R_f = 0.32 (hexane/CHCl₃/2-propanol 15:5:1 by volume).

2-Azido-3-benzoyl-sphingosine- β -Ala

To a stirred solution of 2-azido-3-benzoyl-sphingosine-ONSu (130 mg, 0.228 mmol) in a mixture of CH₂Cl₂ (2 mL) and dimethyl formamide (2 mL) a solution of β -alanine (40.5 mg, 0.455 mmol: 405 μ L of solution 100 mg/mL β -alanine and 86 μ L/mL CF₃COOH in DMSO) and Et₃N (253 μ L, 1.82 mmol) were added, and the mixture was stirred for 19 h at ambient temperature. The reaction mixture was evaporated in vacuum (oil pump) and dried. The residue was extracted in a mixture of CHCl₃ (4 mL), water (4 mL) and 2 M HCl (0.12 mL). Chloroform layer was washed with water (2 x 4 mL), evaporated, and the residue was dried in vacuum. The crude material was purified on silica gel column (~ 80 ml) in hexane/CHCl₃/2-propanol 15:5:1 by volume, elution with hexane/CHCl₃/2-propanol 15:5:1 + 0.5% AcOH by volume. After evaporation of fractions a residue was dissolved in chloroform, the solution was washed with water (2 x 3 mL), diluted with MeCN (3 mL) and evaporated. Thoroughly drying of the residue gave 105.5 mg (85%) of pure 2-azido-3-benzoyl-sphingosine- β -Ala, colorless syrupy glass.

TLC: R_f = 0.12 (hexane/CHCl₃/2-propanol 15:5:1 by volume), R_f = 0.49 (CHCl₃/2-propanol 4:1 by volume).

¹H NMR of 2-azido-3-benzoyl-sphingosine- β -Ala (700 MHz, [D]CHCl₃/[D₄]CH₃OH 1:1, 30°C): δ 8.210 (m, 2H; *ortho*-H of Bz), 7.766 (m, 1H; *para*-H of Bz), 7.636 (m, 2H; *meta*-H of Bz), 6.114 (m, 1H; =CH), 5.754 (m, 2H; CH= and =C-CH-O), 4.383 (dd, J = 11.4, 4.6 Hz, 1H; OCH), 4.274 (dd, J

= 11.4, 7.7 Hz, 1H; OCH⁺), 4.172 (m, 1H; CH-N₃), 3.565 (t, *J* = 6.6 Hz, 2H; NCH₂ of βAla), 2.693 (t, *J* = 6.6 Hz, 2H; CH₂CO of βAla), 2.260 (q, *J* = 7.1, 7.1, 6.8 Hz, 2H; =C-CH₂), 1.565 (m, 2H; =C-C-CH₂), 1.440 (m, 20H; 10 CH₂), 1.043, (t, *J* = 7.1 Hz, 3H; CH₃) ppm.

2-Azido-3-benzoyl-sphingosine-β-Ala-ONSu

To a stirred solution of 2-azido-3-benzoyl-sphingosine-β-Ala (40 mg, 73.4 micromol) in 1,2-dichloroethane (1 mL) a solution of disuccinimidylcarbonate (37.6 mg, 147 micromol: 470 μL of 80 mg/mL solution in dimethyl formamide) and Et₃N (15.3 μL, 110 micromol) were added, and the mixture was stirred for 1.5 h at ambient temperature. The reaction mixture was acidified with AcOH (100 μL) and was placed on Sephadex LH-20 column (column volume 90 mL, eluent – CHCl₃/2-propanol 2:1 + 0.5% AcOH by volume). Fractions, containing pure 2-azido-3-benzoyl-sphingosine-β-Ala-ONSu were combined, evaporated, and the residue was dried in vacuum. Yield of 2-azido-3-benzoyl-sphingosine-β-Ala-ONSu was 42.4 mg (90%), white solid. TLC: R_f = 0.56 (hexane/CHCl₃/2-propanol 15:5:1 by volume).

2-Azido-3-benzoyl-sphingosine-β-Ala-CMG(2)-biot

To a stirred suspension of biot-CMG(2) amine tri-Na salt (44 mg, 34.7 micromol) in dimethyl sulfoxide (2 mL) a solution of 2-azido-3-benzoyl-sphingosine-β-Ala-ONSu (24.5 mg, 38.2 micromol) in 1,2-dichloroethane (0.49 mL), water (0.7 mL) and 1 M aqueous NaHCO₃ (34.7 μL) were added, and the mixture was stirred for 2 h at ambient temperature. The reaction mixture was acidified with AcOH (6 μL) and placed on Sephadex LH-20 column (column volume 130 mL, eluent – water/MeOH/2-propanol/CHCl₃ 40:14:10:1 by volume). Fractions, containing pure 2-azido-3-benzoyl-sphingosine-β-Ala-CMG(2)-biot were combined, evaporated, the residue was dissolved in water (3 mL) and freeze-dried. Yield of 2-azido-3-benzoyl-sphingosine-β-Ala-CMG(2)-biot tetra-Na salt was 56,7 mg (90% on biot-CMG(2) amine), white solid. TLC: R_f = 0.64 (CH₂Cl₂/EtOH/water 1:3:1 by volume), R_f = 0.40 (CH₂Cl₂/EtOH/water 1:3:1 + 2% AcOH by volume).

^1H NMR of 2-azido-3-benzoyl-sphingosine- β -Ala-CMG(2)-biot (700 MHz, $[\text{D}_2]\text{H}_2\text{O}/[\text{D}_4]\text{CH}_3\text{OH}$ 1:1, 30°C): δ 8.035 (m, 2H; *ortho*-H of Bz), 7.653 (m, 1H; *para*-H of Bz), 7.507 (m, 2H; *meta*-H of Bz), 5.951 (m, 1H; =CH), 5.602 (m, 2H; CH= and =C-CH-O), 4.581 (dd, $J = 7.9, 5.0$ Hz, 1H; NHCH of biotin), 4.396 (dd, $J = 7.9, 4.5$ Hz, 1H; NHCH of biotin), 4.333-3.899 (total 35H; 4 CH_2COO , 12 NCH_2CO , CH_2O , CH-N_3), 3.469-3.351 (m, 6H; $\text{NCH}_2\text{CH}_2\text{N}$ and CH_2N of βAla), 3.289 (m, 1H; NHCHCH of biotin), 2.985 (dd, $J = 12.9, 4.8$ Hz, 1H; NHCHCH of biotin), 2.761 (d, $J = 12.9$ Hz, 1H; NHCHCH of biotin), 2.554 (broad t, 2H; CH_2CO of βAla), 2.354 (m, 2H; COCH_2 of biotin), 2.086 (broad, 2H; =C- CH_2), 1.756 (m, 1H; $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}$ of biotin), 1.677 (m, 2H; $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ of biotin), 1.593 (m, 1H; $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}$ of biotin), 1.462 (m, 2H; $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}_2$ of biotin), 1.353 (m, 2H; =C-C- CH_2), 1.224 (m, 20H; 10 CH_2), 0.865 (t, $J = 7.0$ Hz, 3H; CH_3) ppm.

MS (MALDI-TOF): calc. for $\text{C}_{73}\text{H}_{108}\text{N}_{20}\text{O}_{27}\text{S}$ $\text{Mw}_{\text{isotopic}}=1729$, found 1704 [$\text{M}(-\text{N}_2+2\text{H})+\text{H}$], 1730 [$\text{M}+\text{H}$], 1752 [$\text{M}+\text{Na}$], 1768 [$\text{M}+\text{K}$].

2- N_3 -Sphingosine- β -Ala-CMG(2)-biot

To a stirred solution 2-azido-3-benzoyl-sphingosine- β -Ala-CMG(2)-biot (49.1 mg, 27.01 micromol) in water (4.91 mL) methanol (9.82 mL) and Et_3N (0.737 mL) were added, and the mixture was kept for 77 h at ambient temperature. The reaction mixture was evaporated, the residue was thoroughly dried in vacuum and freeze-dried. The obtained 2- N_3 -sphingosine- β -Ala-CMG(2)-biot was used subsequently without purification.

TLC: $R_f = 0.34$ ($\text{CH}_2\text{Cl}_2/\text{EtOH}/\text{water}$ 1:3:1 + 2% AcOH by volume).

^1H NMR of 2- N_3 -Sphingosine- β -Ala-CMG(2)-biot (700 MHz, $[\text{D}_2]\text{H}_2\text{O}/[\text{D}_4]\text{CH}_3\text{OH}$ 1:1, 30°C): δ 5.812 (m, 1H; CH=), 5.518 (m, 1H; =CH), 4.593 (dd, $J = 7.9, 5.0$ Hz, 1H; NHCH of biotin), 4.409 (dd, $J = 7.9, 4.5$ Hz, 1H; NHCH of biotin), 4.272-3.934 (total 35H; 4 CH_2COO , 12 NCH_2CO , CH_2O , CH-N_3), 3.720 (m, 1H; =C-CH-O), 3.459-3.352 (m, 6H; $\text{NCH}_2\text{CH}_2\text{N}$ and CH_2N of βAla), 3.304 (m, 1H; NHCHCH of biotin), 2.998 (dd, $J = 12.9, 4.9$ Hz, 1H; NHCHCH of biotin), 2.769 (d, $J = 12.9$ Hz, 1H; NHCHCH of biotin), 2.557 (t, $J = 6.5$ Hz, 2H; CH_2CO of βAla), 2.360 (m, 2H; COCH_2 of biotin), 2.095 (m, 2H; =C- CH_2), 1.771 (m, 1H; $\text{COCH}_2\text{CH}_2\text{CH}_2\text{CH}$ of biotin), 1.696 (m, 2H;

COCH₂CH₂CH₂CH₂ of biotin), 1.610 (m, 1H; COCH₂CH₂CH₂CH₂ of biotin), 1.469 (m, 2H; COCH₂CH₂CH₂CH₂ of biotin), 1.418 (m, 2H; =C-C-CH₂), 1.281 (m, 20H; 10 CH₂), 0.898 (t, *J* = 7.1 Hz, 3H; CH₃) ppm.

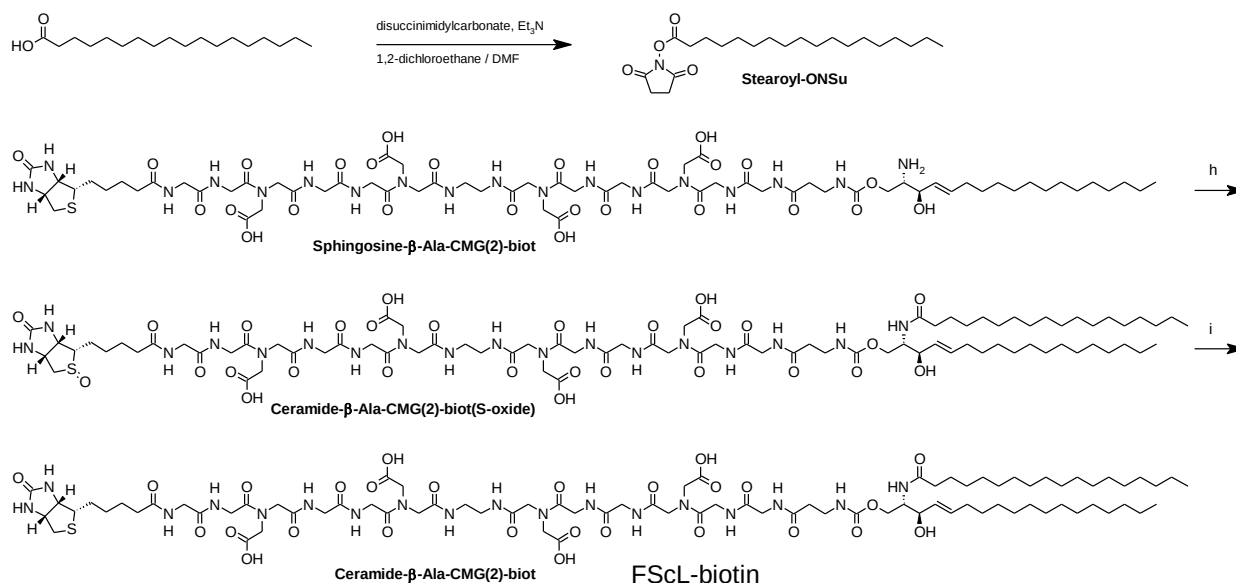
Sphingosine-β-Ala-CMG(2)-biot

To a stirred solution of 2-N₃-sphingosine-β-Ala-CMG(2)-biot (27.01 micromol from previous stage) in water (1.5 mL) methanol (4.5 mL), dithiothreitol (150 mg) and Et₃N (30 μL) were added, and the mixture was stirred for 48 h at ambient temperature. The reaction mixture was evaporated to dryness, the residue was dissolved in 2 mL of water/2-propanol mixture (2:1) and placed on Sephadex LH-20 column (column volume 90 mL, eluent – water/2-propanol 2:1 by volume + 0.05 M Py•HOAc). Fractions, containing pure Sphingosine-β-Ala-CMG(2)-biot were combined, evaporated and the residue was dried in vacuum. Yield of sphingosine-β-Ala-CMG(2)-biot was 44.3 mg (89% on 2-azido-3-benzoyl-sphingosine-β-Ala-CMG(2)-biot, if calculated as tripyridinium salt), white solid.

TLC: 2-N₃-sphingosine-β-Ala-CMG(2)-biot *R_f* = 0.34; sphingosine-β-Ala-CMG(2)-biot *R_f* = 0.29 (CH₂Cl₂/EtOH/water 1:3:1 + 2% AcOH by volume), ninhydrin-positive.

Preparation of Ceramide- β -Ala-CMG(2)-biot

Scheme 1b.3



(h) Stearoyl-ONSu in 1,2-dichloroethane, aq. NaHCO₃, H₂O/2-propanol; (i) N-Methylmercaptoacetamide. H₂O, 69 h at 40 °C.

Stearoyl-ONSu

To a stirred solution of stearic acid (76 mg, 0.27 micromol) in 1,2-dichloroethane (1 mL) and dimethyl formamide (1 mL) mixture disuccinimidylcarbonate (139 mg, 0.54 mmol) and Et₃N (37 μ L, 0.27 mmol) were added. The reaction mixture was stirred for 40 min, acidified with AcOH (60 μ L) and placed on Sephadex LH-20 column (column volume 130 mL, eluent – CHCl₃/2-propanol 2:1 by volume + 0.5% HOAc). Fractions, containing pure stearoyl-ONSu were combined, evaporated and the residue was dried in vacuum; yield 93 mg (91%), white solid.

TLC: stearic acid R_f = 0.49, stearoyl-ONSu R_f = 0.25 (hexane/EtOAc/AcOH 10:2:0.3 by volume).

Ceramide- β -Ala-CMG(2)-biot(S-oxide)

To a stirred solution of sphingosine- β -Ala-CMG(2)-biot (44.3 mg, 24.1 micromol) in a mixture of water (2 mL) and 2-propanol (3 mL) 1 M aqueous NaHCO₃ (160 μ L) and a solution of N-oxysuccinimide ester of stearic acid (20.3 mg, 53 micromol) in 1,2-dichloroethane (0.27 mL) were added, and the

mixture was stirred for 8 h at ambient temperature. Additional portions of N-oxysuccinimide ester of stearic acid (20.3 mg, 53 micromol) in 1,2-dichloroethane (0.27 mL) and 1 M aqueous NaHCO₃ (160 µL) were added, and the mixture was stirred for 15 h at ambient temperature. The reaction mixture was acidified with AcOH (18 µL), evaporated, and the residue was dried in vacuum. The reaction products were separated on silica gel column (75 mL, prepared in CHCl₃/MeOH 4:1 by volume) eluted with CHCl₃/MeOH/water 2:6:1 by volume. Chromatography was accompanied and complicated with self-oxidation of biotin group into biotin(S-oxide). Repeated separation of ceramide-β-Ala-CMG(2)-biot, ceramide-β-Ala-CMG(2)-biot(S-oxide) and minor sphingosine-β-Ala-CMG(2)-biot(S-oxide) (oxidation of unreacted sphingosine-β-Ala-CMG(2)-biot) on silica gel column (75 mL, prepared in CHCl₃/MeOH 4:1 by volume, eluted with CH₂Cl₂/EtOH/water 1:2:1 + 1% Py) gave 20.5 mg (yield 45%) of pure ceramide-β-Ala-CMG(2)-biot(S-oxide).

TLC: ceramide-β-Ala-CMG(2)-biot R_f = 0.62, ceramide-β-Ala-CMG(2)-biot(S-oxide) R_f = 0.54, sphingosine-β-Ala-CMG(2)-biot(S-oxide) R_f = 0.46 (CH₂Cl₂/EtOH/water 1:3:1 + 1% Py by volume).

¹H NMR of ceramide-β-Ala-CMG(2)-biot(S-oxide) (700 MHz, [D₂]H₂O/[D₄]CH₃OH 2:1, 30°C): δ 5.769 (m, 1H; CH=), 5.454 (m, 1H; =CH), under water (NHCH of biotin-S-oxide), 4.721 (dd, J = 8.9, 5.5 Hz, 1H; NHCH of biotin-S-oxide), 4.331-3.897 (total 36H; 4 CH₂COO, 12 NCH₂CO, CH₂O, CHN, =C-CH-O), 3.639 (d, J = 13.5, 1.9 Hz, 1H; NHCHCH of biotin-S-oxide), 3.446-3.344 (m, 7H; NCH₂CH₂N, CH₂N of βAla and NHCHCH of biotin-S-oxide), 3.219 (dd, J = 13.5, 6.7 Hz, 1H; NHCHCH of biotin-S-oxide), 2.536 (broad t, 2H; CH₂CO of βAla), 2.394 (t, J = 7.3 Hz, 2H; COCH₂ of biotin-S-oxide), 2.208 (broad t, 2H; COCH₂ of stearyl), 2.030 (m, 2H; =C-CH₂), 1.901 (m, 2H; COCH₂CH₂ of stearyl), 1.734 and 1.593 (m, 6H; 3CH₂ of biotin-S-oxide), 1.304 (broad s, 50H; 25CH₂), 0.902 (t, J = 6.9 Hz, 3H; CH₃), 0.894 (broad t, J = 7.1 Hz, 3H; CH₃) ppm.

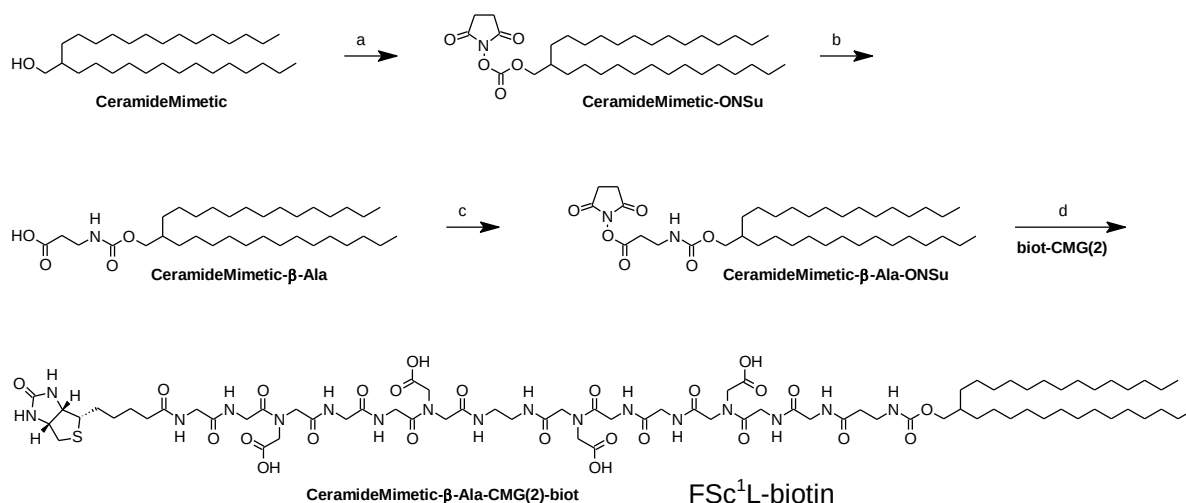
MS (MALDI-TOF): calc. for C₈₄H₁₄₀N₁₈O₂₈S M_w_{isotopic}=1881, found 1882 [M+H], 1904 [M+Na], 1920 [M+K].

Ceramide- β -Ala-CMG(2)-biot (FScL-biotin)

To a stirred solution of ceramide- β -Ala-CMG(2)-biot(S-oxide) (20.5 mg, 10.89 micromol) in water (1.3 mL) N-methyl-mercaptoacetamide (373 μ L) was added, and the mixture was kept for 69 h at 40 °C. The reaction mixture was placed on Sephadex LH-20 column (column volume 90 mL, eluent – water/2-propanol 2:1 by volume + 0.03 M HOAc + 0.06 M Py). Fractions, containing pure ceramide- β -Ala-CMG(2)-biot were combined, evaporated, and the residue was thoroughly dried in vacuum. The residue was dissolved in water (1 mL), titrated to pH 6.5 with 0.1 M NaHCO₃ and freeze-dried. Yield of ceramide- β -Ala-CMG(2)-biot tetra-Na salt was 15.8 mg (74%), white solid. TLC: ceramide- β -Ala-CMG(2)-biot R_f = 0.64 (CH₂Cl₂/EtOH/water 1:3:1 by volume).

¹H NMR of ceramide- β -Ala-CMG(2)-biot (700 MHz, [D₂]H₂O/[D₄]CH₃OH 2:1, 30°C): δ 5.764 (m, 1H; CH=), 5.443 (m, 1H; =CH), 4.603 (dd, *J* = 7.9, 4.9 Hz, 1H; NHCH of biotin), 4.423 (dd, *J* = 7.9, 4.6 Hz, 1H; NHCH of biotin), 4.312-3.964 (total 36H; 4 CH₂COO, 12 NCH₂CO, CH₂O, CHN and =C-CH-O), 3.444-3.352 (m, 6H; NCH₂CH₂N and CH₂N of β Ala), 3.313 (m, 1H; NHCHCH of biotin), 2.997 (dd, *J* = 12.9, 4.8 Hz, 1H; NHCHCH of biotin), 2.778 (d, *J* = 12.9 Hz, 1H; NHCHCH of biotin), 2.552 (t, *J* = 6.5 Hz, 2H; CH₂CO of β Ala), 2.365 (m, 2H; COCH₂ of biotin), 2.206 (broad t, 2H; COCH₂ of stearyl), 2.039 (m, 2H; =C-CH₂), 1.759, 1.691, 1.601, 1.558 and 1.469 (m, total 8H; 6H of biot and COCH₂CH₂ of stearyl), 1.390 (m, 2H; =C-C-CH₂), 1.299 (m, 48H; 24 CH₂), 0.905 (t, *J* = 6.6 Hz, 3H; CH₃), 0.895 (broad t, *J* = 7.0 Hz, 3H; CH₃) ppm. MS (MALDI-TOF): calc. for C₈₄H₁₄₀N₁₈O₂₇S Mw_{isotopic}=1865, found 1866 [M+H], 1888 [M+Na], 1904 [M+K].

Supplementary Note 1c. **Synthetic scheme and detailed protocol for synthesis of FSc¹L-biot (mimetic of FScL-biot)**



(a) disuccinimidylcarbonate, Et₃N, DMF/CH₂Cl₂ (b) β-alanine, Et₃N, DMSO/DMF/1,2-dichloroethane; (c) disuccinimidylcarbonate, Et₃N, DMF/1,2-dichloroethane; (d) biot-CMG(2) amine (see Supplementary Note 1b), aq. NaHCO₃, 1,2-dichloroethane/DMSO/H₂O.

CeramideMimetic-ONSu

To a stirred solution of CeramideMimetic (2-(tetradecyl)hexadecanol, *Katayama Chemical Industries Co., Ltd.*, 15.3 mg, 34.9 micromol) in a mixture of CH₂Cl₂ (1 mL) and dimethyl formamide (0.6 mL) a solution of disuccinimidylcarbonate (72 mg, 0.28 mmole) in dimethyl formamide (0.89 mL) and Et₃N (19.5 μL, 0.14 mmole) were added, and the mixture was stirred for 24 h at ambient temperature. The reaction mixture was acidified with AcOH (96 μL) and was placed on Sephadex LH-20 column (column volume 90 mL, eluent – CHCl₃/2-propanol 2:1 + 0.5% AcOH by volume). Fractions, containing pure CeramideMimetic-ONSu were combined, evaporated, and the residue was dried in vacuum. Yield of CeramideMimetic-ONSu was 19.1 mg (94%), white solid.

TLC: R_f = 0.35 (hexane/CHCl₃/2-propanol 15:5:1 by volume).

CeramideMimetic- β -Ala

To a stirred solution of CeramideMimetic-ONSu (19.1 mg, 32.9 micromol) in a mixture of 1,2-dichloroethane (1 mL) and dimethyl formamide (2 mL) a solution of β -alanine (10.6 mg, 119 micromol: 106 μ L of solution 100 mg/mL β -alanine and 86 μ L/mL CF_3COOH in DMSO) and Et_3N (66 μ L, 0.47 mmol) were added, and the mixture was stirred for 17 h at ambient temperature. The reaction mixture was acidified with AcOH (60 μ L), evaporated with 5 mL of 2-propanol to minimum volume and was placed on Sephadex LH-20 column (column volume 90 mL, eluent – CHCl_3 /2-propanol 2:1 by volume + 0.05 M $\text{Py}\cdot\text{AcOH}$). Fractions, containing pure CeramideMimetic- β -Ala were combined, evaporated, and the residue was dried in vacuum. Yield of CeramideMimetic- β -Ala was 17.4 mg (95%), white solid.

TLC: R_f = 0.38 (hexane/ CHCl_3 /2-propanol 2:4:1 by volume).

^1H NMR of CeramideMimetic- β -Ala (700 MHz, $[\text{D}]\text{CHCl}_3/[\text{D}_4]\text{CH}_3\text{OH}$ 1:1, 30°C): δ 4.098 (d, J = 5.4 Hz, 2H; CH_2O), 3.549 (t, J = 6.5 Hz, 2H; CH_2N of β -Ala), 2.684 (t, J = 6.5 Hz, 2H; CH_2CO of β -Ala), 1.766 (m, 1H; OCH_2CH), 1.433 (m, 52H; 26 CH_2), 1.046, (t, J = 7.1 Hz, 6H; 2 CH_3) ppm.

CeramideMimetic- β -Ala-ONSu

To a stirred solution of CeramideMimetic- β -Ala (11.3 mg, 20.4 micromol) in a mixture of 1,2-dichloroethane (1 mL) and dimethyl formamide (0.35 mL) a solution of disuccinimidylcarbonate (10.5 mg, 41 micromol: 131 μ L of 80 mg/mL solution in dimethyl formamide) and Et_3N (4.3 μ L, 31 micromol) were added, and the mixture was stirred for 2 h at ambient temperature. The reaction mixture was acidified with AcOH (100 μ L) and was placed on Sephadex LH-20 column (column volume 90 mL, eluent – CHCl_3 /2-propanol 2:1 + 0.5% AcOH by volume). Fractions, containing pure CeramideMimetic- β -Ala-ONSu were combined, evaporated, and the residue was dried in vacuum. Yield of CeramideMimetic- β -Ala-ONSu was 12.7 mg (96%), white solid.

TLC: R_f = 0.72 (hexane/ CHCl_3 /2-propanol 2:4:1 by volume).

CeramideMimetic- β -Ala-CMG(2)-biot (FSc¹L-biot)

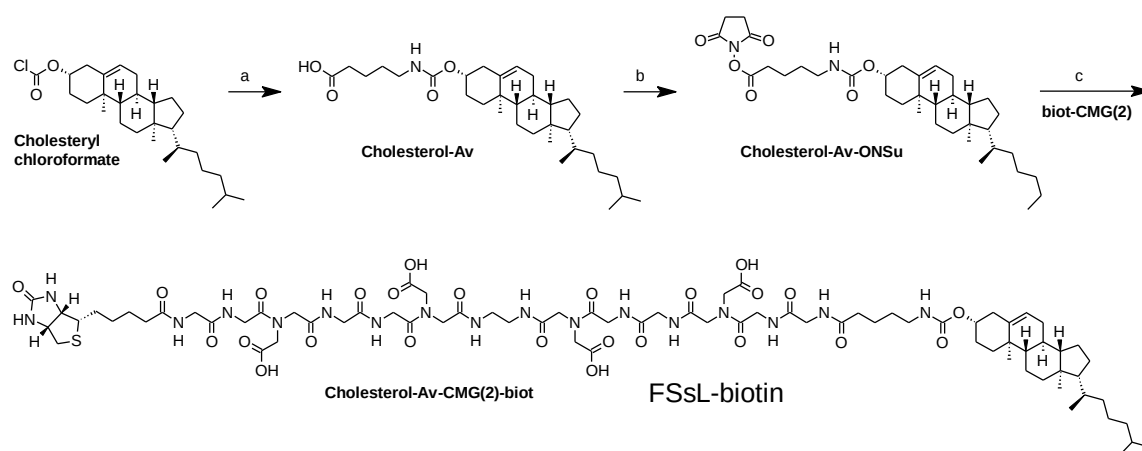
To a stirred suspension of biot-CMG(2) amine tri-Na salt (22.3 mg, 17.6 micromol, described in Supplementary Note 1b) in dimethyl sulfoxide (1.5 mL) a solution of CeramideMimetic- β -Ala-ONSu (12.7 mg, 19.5 micromol) in 1,2-dichloroethane (0.25 mL), water (0.35 mL) and 1 M aqueous NaHCO₃ (35.2 μ L) were added, and the mixture was stirred for 5 h at ambient temperature. The reaction mixture was acidified with AcOH (3 μ L), evaporated with 3 mL of 2-propanol/water 1:1 mixture to minimum volume and was placed on Sephadex LH-20 column (column volume 90 mL, eluent – 2-propanol/water 1:2 + 3% CH₂Cl₂ + 0.3% Py by volume). Fractions, containing pure CeramideMimetic- β -Ala-CMG(2)-biot were combined, evaporated, and the residue was thoroughly dried in vacuum. The residue was dissolved in water (1 mL), titrated to pH 6.5 with 0.1 M NaHCO₃ and freeze-dried. Yield of CeramideMimetic- β -Ala-CMG(2)-biot tetra-Na salt was 29.2 mg (91% on biot-CMG(2) amine), white solid.

TLC: R_f = 0.61 (CHCl₃/CH₃OH/water 2:6:1 by volume).

¹H NMR of CeramideMimetic- β -Ala-CMG(2)-biot (700 MHz, [D₂]H₂O/[D₄]CH₃OH 1:1, 30°C): δ 4.590 (dd, *J* = 7.9, 4.7 Hz, 1H; NHCH of biotin), 4.408 (dd, *J* = 7.9, 4.6 Hz, 1H; NHCH of biotin), 4.293-3.917 (total 34H; 4 CH₂COO, 12 NCH₂CO, CH₂O), 3.428-3.343 (m, 6H; NCH₂CH₂N and CH₂N of β Ala), 3.302 (m, 1H; NHCHCH of biotin), 2.993 (dd, *J* = 12.9, 5.0 Hz, 1H; NHCHCH of biotin), 2.767 (d, *J* = 12.9 Hz, 1H; NHCHCH of biotin), 2.546 (t, *J* = 6.5 Hz, 2H; CH₂CO of β Ala), 2.359 (m, 2H; COCH₂ of biotin), 1.768, (m, 1H; COCH₂CH₂CH₂CH of biotin), 1.695 (m, 2H; COCH₂CH₂CH₂CH₂ of biotin), 1.609 (m, 2H; COCH₂CH₂CH₂CH of biotin and OCH₂CH), 1.468 (m, 2H; COCH₂CH₂CH₂CH₂ of biotin), 1.312 (m, 52H; 26 CH₂), 0.919, (t, *J* = 7.0 Hz, 6H; 2 CH₃) ppm.

MS (MALDI-TOF): calc. for C₇₈H₁₃₁N₁₇O₂₅S Mw=1739, found 1740 [M+H], 1762 [M+Na], 1778 [M+K].

Supplementary Note 1d. **Synthetic scheme and detailed protocol for synthesis of FSsL-biot.**



(a) 5-aminovaleric acid, $\text{CHCl}_3/\text{H}_2\text{O}$, NaHCO_3 ; (b) disuccinimidylcarbonate, Et_3N , $\text{DMF}/1,2\text{-dichloroethane}$; (c) biot-CMG(2) amine (see Supplementary Note 1b), aq. NaHCO_3 , $1,2\text{-dichloroethane}/\text{DMSO}/\text{H}_2\text{O}$.

Cholesterol-aminovaleric (AV) acid

To a rapidly stirred solution of 5-aminovaleric acid (117 mg, 1 mmol) and NaHCO_3 (252 mg, 3 mmol) in water (10 ml) a solution of cholesteryl chloroformate (449 mg, 1 mmol) in CHCl_3 (10 ml) was added. The mixture was stirred for 4 h at r.t., then acidified with 1 M HCl (2.5 ml) and chloroform layer was separated. After additional extraction of aqueous solution with chloroform (2 x 10 ml) combined organic extracts were washed with saturated NaCl and evaporated. The crude product was purified on silica gel column in $\text{CHCl}_3/\text{EtOAc}$ 4:1 + 1% AcOH mixture as eluent. Yield of cholesteryl-carbonylaminopentanoic acid was 434 mg (82%), white solid.

TLC: $R_f = 0.35$ ($\text{CHCl}_3/\text{EtOAc}$ 3:1 + 1% AcOH).

^1H NMR (700 MHz, $[\text{D}]\text{CHCl}_3$, 30°C): 9.95 (broad. s, 1H; COOH), 5.372 (m, 1H; $=\text{CH}$), 4.647 (m, 1H; NH), 4.490 (m, 1H; OCH), 3.187 (m, 2H; CH_2N), 2.387 (t, 2H; CH_2CO), 2.359 (m, 1H; $=\text{C}-\text{CH}$), 2.264 (m, 1H, $=\text{C}-\text{CH}'$), 1.982 (m, 2H), 1.846 (m, 3H), 1.677 (m, 2H), 1.599-1.304 (m, 12H), 1.257 (m, 1H), 1.188-0.926 (m, 13H), 0.916 (d, $J = 6.5$ Hz, 3H; CH_3), 0.868 (d, $J = 6.6$ Hz, 3H; CH_3), 0.864 (d, $J = 6.6$ Hz, 3H; CH_3), 0.678 (s, 3H; CH_3) ppm.

Cholesterol-AV-ONSu

To a solution of cholesterol-AV (57 mg, 0.108 mmol) in the mixture of 1,2-dichloroethane (0.6 mL) and DMF (1.2 mL) disuccinimidyl carbonate (55 mg, 0.215 mmol) and Et₃N (15 μ L, 0.108 mmol) were added. The solution was stirred for 30 min and then was acidified with AcOH (37 μ L). Gel-permeation chromatography on Sephadex LH-20 (~ 90 mL of gel in CHCl₃/MeOH 2:1 + 0.5% AcOH), evaporation of fractions and drying of the residue in vacuum gave 67 mg (~ quantitative yield) of pure cholesteroylcarbonylaminopentanoic acid oxisuccinimide ester.

TLC: R_f = 0.29 (hexane/ethyl acetate/AcOH 10:4:1).

biot-CMG(2)-AV-cholesterol (FSsL-biot)

biot-CMG(2) amine tri-Na salt (68 mg, 0.0536 mmol, described in Supplementary Note 1b) was dissolved in water (3.8 mL) and diluted with 2-propanol (4.2 mL). To the intensively stirred solution a solution of cholesterol-AV-ONSu (67 mg, 0.107 mmol) in a mixture of 1,2-dichloroethane (1.1 mL) and DMF (3 mL) and then aqueous 1M NaHCO₃ (0.59 mL) were added. After 30 min of stirring (room temperature) 3 mL of mixture 2-propanol/water (1:1) was added, and the solution was stirred for additional 30 min. The reaction mixture was acidified with AcOH (68 μ L), evaporated, and a residue was dried in vacuum. The obtained residue was washed with fresh-distillated Et₂O (3 x 8 mL, decantation) to remove excessive cholesterol-AV-ONSu and DMF. Gel-permeation chromatography of the crude product on Sephadex LH-20 (~ 170 mL of gel in 2-propanol/water 1:2), evaporation of fractions and drying of the residue in vacuum gave pure product. Freeze-drying of aqueous solution of product (2.5 mL of water) gave 86 mg (89% calc. on biot-CMG(2) amine) of biot-CMG(2)-AV-cholesterol tetra-Na salt.

TLC: R_f = 0.51 (CHCl₃/MeOH/water 2:6:1).

¹H NMR (700 MHz, [D₂]H₂O/[D₄]CH₃OH 1:2, 30°C): 5.575 (m, 1H; =CH of cholesterol), 4.737 (dd, *J* = 7.8 Hz, *J* = 5 Hz, 1H; NHCH of biotin), 4.553 (dd, *J* = 7.8 Hz, *J* = 4.3 Hz, 1H; NHCH of biotin), 4.534 (m, 1H; OCH of cholesterol), 4.470-4.078 (total 32H; 4 CH₂COO, 12 NCH₂CO), 3.540 (m, 4H; NHCH₂CH₂NH), 3.446 (m, 1H; NHCHCH of biotin), 3.290 (t, *J* = 6.7 Hz, 2H;

CH₂N of aminovaleric res.), 3.149 (dd, $J = 13$ Hz, $J = 5$ Hz, 1H; NHCHCH of biotin), 2.916 (dd, $J = 13$ Hz, $J = 2$ Hz, 1H; NHCHCH of biotin), 2.515 (m, 6H; 2 CH₂CO and =CH-CH₂ of cholesterol), 2.208-1.105 (total 39H; 29 H of cholesterol, 4H of aminovaleric res. and 6H of biotin), 1.105 (d, $J = 6.6$ Hz, 3H; CH₃CH of cholesterol), 1.050 and 1.046 (2 d, $J = 6.6$ Hz, 2x3H; 2 CH₃CH of cholesterol), 0.878 (s, 3H; CH₃ of cholesterol) ppm.

MS (MALDI-TOF): calc. for C₇₇H₁₁₉N₁₇O₂₅S $Mw_{isotopic}$ =1714, found 1737 [M+Na], 1759 [MNa+Na].

Supplementary references

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