# Modulating efficacy and cytotoxicity of lipoamino fatty acid nucleic acid carriers by disulfide or hydrophobic spacers

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## SUPPORTING INFORMATION

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# 1 Supporting Experimental Procedures

## 1.1 Materials

8-Aminooctanoic acid, glacial acetic acid, cystamine dihydrochloride, dodecanal, *N,N*-diisopropylethylamine (DIPEA), glutathione reduced (GSH), octanal, potassium permanganate, sodium citrate tribasic dihydrate, sodium cyanoborohydrate ($NaBH_3CN$), succinic anhydride and super-DHB (9:1 mixture of 2,5-dihydrocybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) were obtained from Sigma Aldrich (Munich, Germany). Sodium sulfate was purchased from ORG Laborchemie (Bunde, Germany) and N-(9*H*-Fluoren-9-ylmethoxycarbonyloxy)-succinimide (Fmoc-OSu) from Iris Biotech (Marktredwitz, Germany). Acetonitrile, dichloromethane (DCM), dry methanol (MeOH), ethyl acetate (EtOAc), n-heptane, aqueous sodium hydroxide solution (1M) and tetrahydrofuran (THF) were purchased from Thermo Fisher Scientific (Schwerte, Germany). Solid-phase synthesis reactors (polypropylene reactors with polytetrafluoroethylene (PTFE) frits and Luer stopper) were purchased from Multi-SynTech (Witten, Germany). 2-Chlorotrityl chloride resin, dimethylformamide (DMF), piperidine, Fmoc-L-Lysine(Fmoc)-OH, Fmoc-L-Lysine(Dde)-OH, Fmoc-amino butanoic acid and Fmoc-amino hexanoic acid were obtained from Iris Biotech (Marktredwitz, Germany), benzotriazole-1-yl-oxytrispyrrolidino phosphonium hexafluorophosphate (PyBOP), 1-hydroxybenzotriazole (HOBt), di-tert-butyl dicarbonate (Boc anhydride), hydrazine monohydrate, phenol, potassium cyanide and triisopropylsilane (TIS) from Sigma Aldrich (Munich, Germany). Ninhydrin was purchased from AppliChem (Darmstadt, Germany), pyridine and trifluoro acetic acid from Thermo Fisher Scientific (Schwerte, Germany) and ethanol absolute (EtOH) and aqueous hydrochloride solution (1M) from VWR (Darmstadt, Germany). Deuterated solvents were purchased from Eurisotop (Fluorochem, Hadfield, UK). Plasmid pCMVLuc (encoding *Photinus pyralis* firefly luciferase under the control of a cytomegalovirus promoter and enhancer) was obtained from Plasmid Factory GmbH (Bielefeld, Germany), CleanCap® Fluc mRNA (5 moU) and CleanCap EGFP mRNA (5moU) from Trilink Biotechnologies (San Diego, CA, USA) and EZ Cap™ Cy5 Firefly Luciferase mRNA (5-moUTP) from ApexBio (Apexbt Technology LLC, Houston, USA). Agarose BioReagent low EEO, boric acid, bromophenol blue, ethidium bromide (EtBr) (1% solution in $H_2O$), glycerol, RNase-free water, tris(hydroxymethyl) aminomethane hydrochloride (Tris-HCl) and triton X-100 were purchased from Sigma Aldrich (Munich, Germany), 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES) from Biomol (Hamburg, Germany), GelRed (1000x) from VWR (Darmstadt, Germany) and D-(+)-glucose monohydrate and ethylene diaminetetraacetic acid (EDTA) from Merck (Darmstadt, Germany). Quant-iT™ RiboGreen RNA Assay-Kit was obtained from Thermo Fisher Scientific (Schwerte, Germany) and heparin (5000 I.U. $mL^{-1}$) from B. Braun SE (Melsungen, Germany). Human blood was provided by the

hospital of the Ludwig-Maximilians-Universität (Munich, Germany). All cell culture consumables were purchased from Faust Lab Science (Klettgau, Germany). HeLa cells (human adherent cervix carcinoma cell line) were obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany), N2a cells (murine neuroblastoma cell line Neuro2a) from the American Type Culture Collection (ATCC, Manassas, VA, USA) and human adherent hepatic carcinoma cell lines Huh7 wild-type from the Japanese Collection of Research Bioresources Cell Bank (Osaka, Japan). Dulbecco's Modified Eagle's medium (DMEM) low glucose, DMEM Ham's F12 medium, RPMI 1640 medium containing L-glutamine and sodium bicarbonate, fetal calf serum (FCS), stable glutamine, penicillin (100 U $mL^{-1}$) and streptomycin (100 µg $mL^{-1}$) were purchased from Sigma Aldrich (Munich, Germany) and PAN Biotech (Aidenbach, Germany). Adenosine 5'-triphosphate (ATP) disodium salt trihydrate was purchased from Roche Diagnostics GmbH (Mannheim, Germany), coenzyme A trilithium salt, dithiothreitol, glycylglycine, magnesium chloride ($MgCl_2$), para-formaldehyde and 4',6-diamidine-2'-phenylindole dihydrochloride (DAPI) were obtained from Sigma Aldrich (Munich, Germany). Cell lysis buffer 5x, luciferin sodium salt, CellTiter Glo® Luminescent Cell Viability Assay Kit and CytoTox96® non-radioactive cytotoxicity assay kit (LDH release assay) were purchased from Promega (Mannheim, Germany). Bafilomycin A1 and Collagen A were obtained from Merck KGaA (Darmstadt, Germany). µ-Slides 8 well ibidiTreat were obtained from ibidi GmbH (Gräfelfing, Germany) and TACS Annexin V-FITC Apoptosis Detection Kit from Bio-Techne GmbH (Wiesbaden, Germany). LPEI 22 kDa and branched succinylated PEI (succPEI) were synthesized as reported previously.[1, 2] Starting materials (poly(2-ethyl-2-oxazoline for linear PEI and branched PEI 25 kDa for succPEI) were obtained from Sigma Aldrich (Munich, Germany). The syntheses of the disulfide building block (N-Fmoc, N´-succinoyl-cystamine, 'ssbb') and the lipoamino fatty acids 8Oc and 12Oc were performed analogously as described in Klein et al[3] and Thalmayr et al.[4] N-Fmoc, tri-Boc protected succinoyl tetraethylene pentamine (Stp) was prepared by in house synthesis according to previously published protocols.[4, 5]

## 1.2 Analytical methods

### *NMR*

$^1$H-NMR spectra were recorded by using a Bruker Avance III HD 400 (400 MHz). Signals were calibrated to the residual, non-deuterated signals of the used solvent as an internal standard ($CDCl_3$ 7.26 ppm; methanol-$d_4$ 4.87 (s) and 3.31 (p)). Chemical shifts (δ) were reported in parts per million (ppm). The spectra were analyzed by MestreNova (MestReLab Research x64, Version 10.0). Integration was performed manually. Multiplicities were abbreviated as follows: s, singlet; d, doublet; t, triplet; m, multiplet.

***ESI-MS***

ESI-MS was performed using a Thermo Scientific LTQ FT Ultra Fourier transform ion cyclotron and an IonMax source. Data is either shown as $[M+X]^z$ after positive ionization or $[M-X]^{-z}$ after negative ionization. Samples were kindly processed by Dr. Werner Spahl and colleagues from the analytical core facility at the Department of Chemistry, LMU Munich.

***MALDI-TOF***

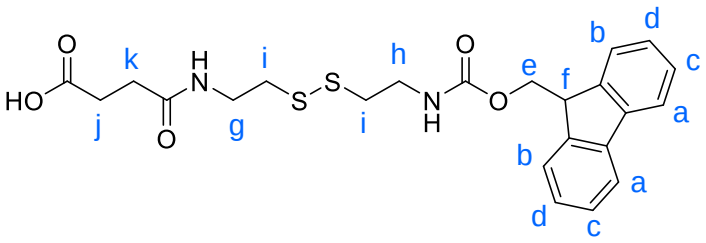
MALDI-TOF mass spectrometry was conducted using an Autoflex II mass spectrometer (Bruker Daltonics, Germany). As matrix, a solution of 10 mg $mL^{-1}$ super-DHB (9/1 (w/w) mixture of 2,5-dihydroxybenzoic acid and 2-hydroxy-5-methoxybenzoic acid) in 69.93/30/0.07 (v/v/v) $H_2O$/ACN/TFA was used. 1 µL of matrix solution was spotted on a MTP AnchorChip (Bruker Daltonics, Germany). Subsequently, 1 µL of sample solution dissolved in $H_2O$ at a concentration of 1 mg $mL^{-1}$, was added onto the matrix, co-crystallized and analyzed. Spectra were recorded in positive ion mode.

For MALDI-TOF analysis under reductive stress, the carrier was diluted with $H_2O$ to a concentration of 1 mg $mL^{-1}$ . 50 mM glutathione (GSH) stock solution was prepared in HBG, and pH was adjusted to 7.4 by addition of 1 M $NaOH_{aq}$. GSH stock solution was added to the oligomer solution to reach a final GSH concentration of 10 mM. The solution was incubated for 90 min at 37°C while shaking at 500 rpm. Afterwards, the sample was prepared and analyzed as described above.

## 1.3 Building block syntheses

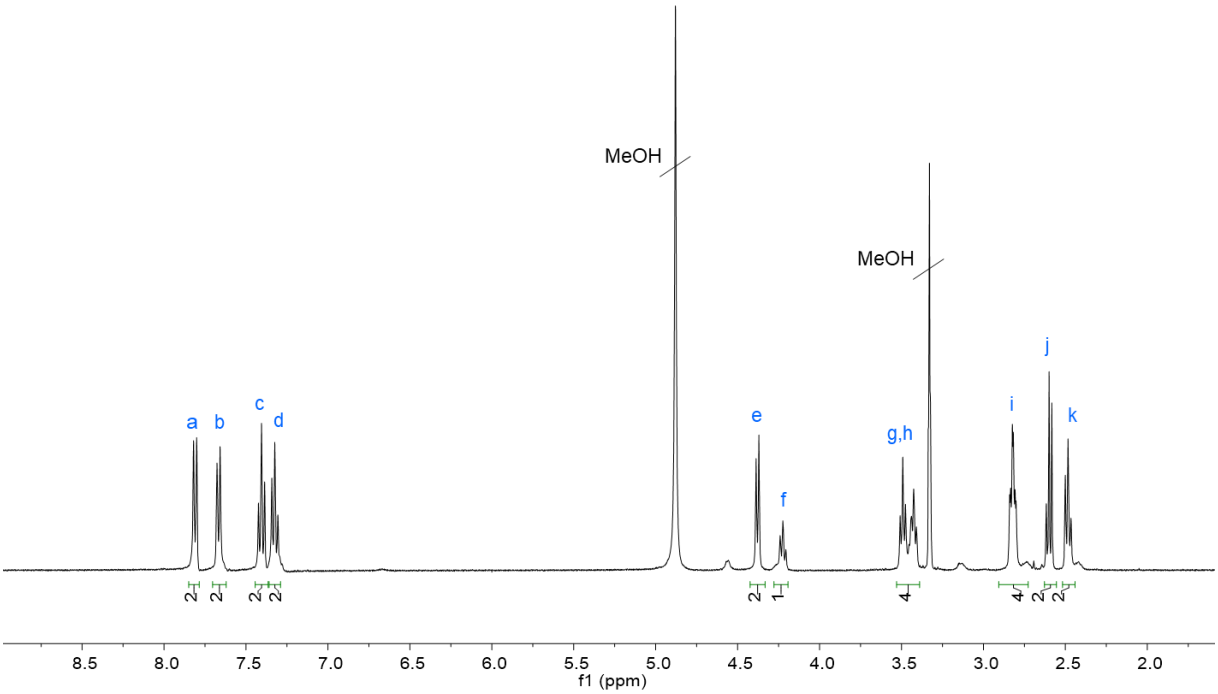
### Disulfide building block 1-(9H-fluoren-9-yl)-3,12-dioxo-2-oxa-7,8-dithia-4,11-diaza-pentadecan-15-oic acid

The synthesis of the disulfide building block was performed analogously as described in Klein et al.[3] In detail, 5.0 g of cystamine dihydrochloride (22.2 mmol, 1 eq.) were suspended in 50 mL of THF with 7.73 mL of DIPEA (44.4 mmol, 2 eq.) and cooled down to −78 °C. 6.0 g (17.8 mmol, 0.8 eq.) of Fmoc-OSu were dissolved in 70 mL of THF and added dropwise over the course of 3 h. The reaction was stirred for additional 1 hour at −78 °C, followed by stirring for 1 h at RT. Then, DIPEA (7.73 mL, 44.4 mmol, 2 eq.) was added and the reaction mixture was cooled to 0 °C. Succinic anhydride (4.0 g, 40 mmol, 1.8 eq.) was dissolved in 50 mL of THF. This solution was added dropwise to the reaction mixture at 0 °C and stirred overnight. The reaction mixture was filtered and concentrated to approximately 200 mL, mixed with 200 mL of DCM and was washed 5× with 0.1 M sodium citrate buffer (pH 5.2). The organic phase was dried over sodium sulfate, concentrated, and purified by silica column chromatography using a n-heptane/EtOAc gradient (starting from 1:1 (v/v)) to elute Fmoc-byproducts, followed by a EtOAc/MeOH gradient to isolate the product. The solvent was removed under reduced pressure to give 2.95 g of a white solid (6.22 mmol, 28%).




$^{1}$HNMR (400 MHz, Methanol-$d_4$) $\delta_H$ (ppm) = 7.81 (d, *J* = 7.5 Hz, 2H, a), 7.67 (d, *J* = 7.5 Hz, 2H, b), 7.41 (d, *J* = 7.4 Hz, 2H, c), 7.37 – 7.28 (m, 2H, d), 4.38 (d, *J* = 6.9 Hz, 2H, e), 4.22 (t, *J* = 6.9 Hz, 1H, f), 3.46 (dt, *J* = 26.6, 6.6 Hz, 4H, g, h), 2.82 (td, *J* = 6.8, 2.8 Hz, 4H, i), 2.60 (dd, *J* = 7.3, 6.1 Hz, 2H, j), 2.48 (t, *J* = 6.9 Hz, 2H, k).

ESI-MS: calculated for $C_{23}H_{26}N_2O_5S_2$ = 474.128; found: $[M+Na]^+$ = 497.118 and $[M-H]^-$ = 473.121

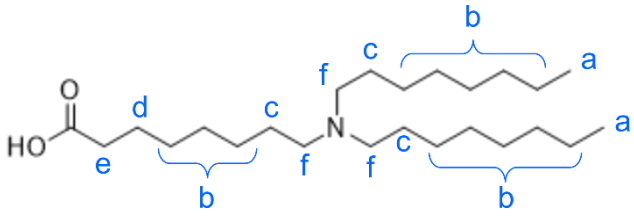



**Synthesis of Lipo amino fatty acids (LAFs) 8Oc and 12Oc**

The protocol was adapted from Thalmayr et al.[4] Briefly, 400 mg of 8-amino octanoic acid was dissolved in 50 mL of dry methanol (MeOH) and stirred for 15 min at RT. Then, 2.2 eq of the indicated fatty aldehyde (octanal and dodecanal, respectively), 2.5 eq of sodium cyanoborohydride ($NaBH_3CN$), and 0.8 eq acetic acid were added successively. The mixture was stirred for 48 hours at RT, before the solvent was removed under reduced pressure and the residual colorless oil was dried under high vacuum. Afterwards, excess of reducing agent was removed by re-dissolving the crude product in DCM and filtration. The filtrate was

concentrated and purified by silica gel chromatography with a solvent gradient from 50:1 to 30:1 to remove unreacted starting material and by-products and 20:1 DCM/MeOH (v/v) to isolate the desired product. Elution of products was detected by thin-layer chromatography and staining by basic potassium permanganate ($KMnO_4$) solution and careful warming. Products were characterized by ESI-MS (electrospray ionization mass spectrometry) and $^1$H-NMR. Analytical data are in accordance with literature.[4]

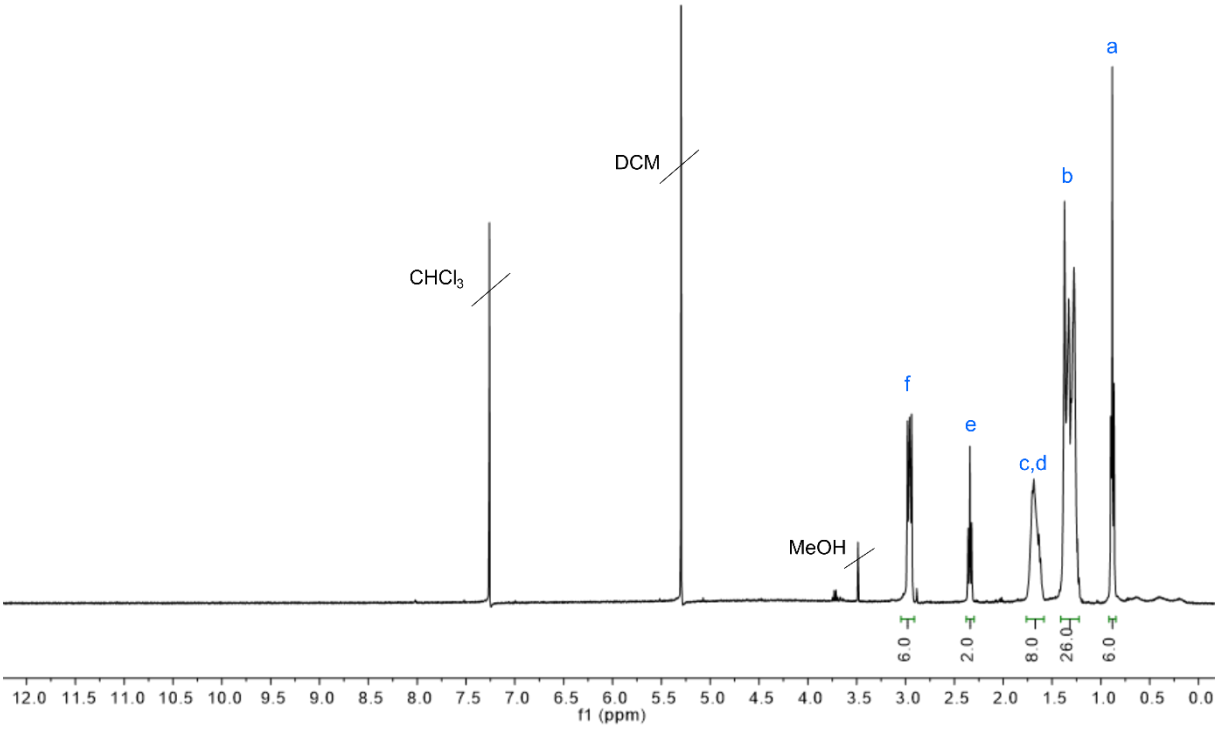
**LAF 8Oc**



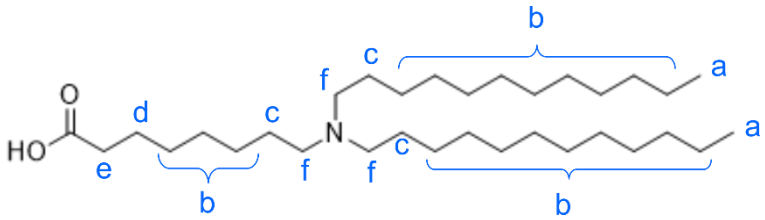

Yield: 320.9 mg, 33%

$^1$H NMR (400 MHz, Chloroform-*d*) $\delta_H$ (ppm) = 3.03 – 2.93 (m, 6H, f), 2.34 (t, *J* = 7.3 Hz, 2H, e), 1.67 (t, *J* = 14.1 Hz, 8H, c, d), 1.42 – 1.21 (m, 28H, b), 0.92 – 0.84 (m, 6H, a).

ESI-MS: calculated for $C_{24}H_{50}NO_2$ $[M+H]^+$ = 384.38361; found $[M+H]^+$ = 384.38325



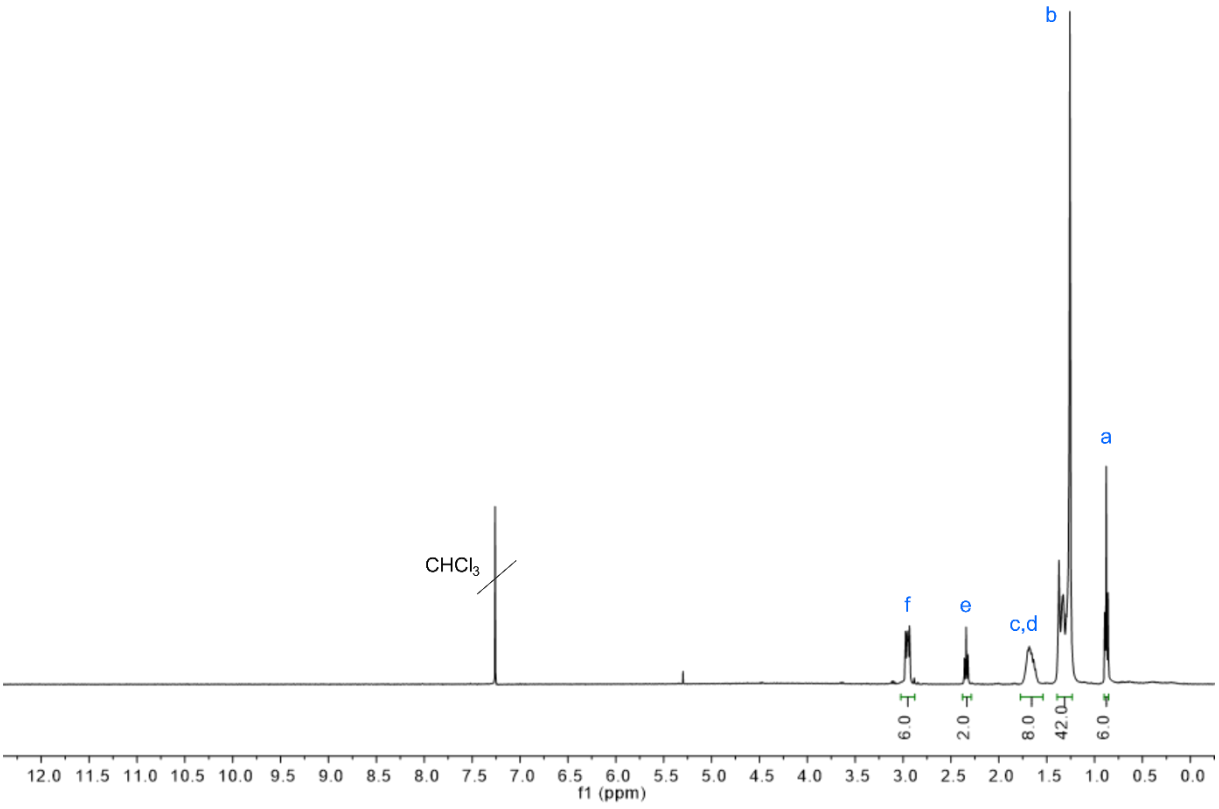
**LAF 12Oc**




Yield: 623.7 mg, 51 %;

$^{1}$H NMR (400 MHz, Chloroform-*d*) $\delta_H$ (ppm) = 3.01 – 2.91 (m, 6H, f), 2.34 (t, *J* = 7.3 Hz, 2H, e), 1.66 (dt, *J* = 21.2, 7.8 Hz, 8H, c, d), 1.31 (d, *J* = 45.2 Hz, 42H, b), 0.91 – 0.84 (m, 6H, a).

ESI-MS: calculated for $C_{32}H_{66}NO_2$ $[M+H]^+$ = 496.50881; found $[M+H]^+$ = 496.51



### 1.4 General procedure for solid phase synthesis

**Resin loading.** The LAF containing carriers were synthesized by solid phase assisted synthesis using an acid-labile 2-chlorotrityl resin (theoretical loading: 1.55 mmol $g^{-1}$). 50 mg of resin were transferred to a syringe reactor and swelled in dry DCM for 20 min. The solvent was ejected and the first amino acid or building block (0.3 eq) was dissolved in dry DCM. After addition of 0.9 eq DIPEA, the solution was immediately added to the resin and allowed to react for 60 minutes at RT while shaking. Afterwards, free reaction sites were capped. Therefore, the resin was incubated with a capping solution consisting of 1 mL dry DCM, 0.75 mL MeOH and 0.125 mL DIPEA to the resin for 30 min. Then, the solution was discarded, and the resin was washed with DMF and DCM (5 x 2 mL each) and dried under reduced pressure. Resin loading was determined by UV/Vis spectrometry by detection of the absorbance of free fulvene product resulting from Fmoc deprotection. Therefore, 5-10 mg of the resin was treated with 1 mL of Fmoc deprotection solution consisting of 20% piperidine in DMF (v/v) for 60 min while shaking at 500 rpm. After centrifugation at 10,000 rpm for 20 sec, 25 µL of the supernatant were diluted in 975 µL of DMF, transferred to a cuvette and absorbance was measured by a multiwell photometer at 301 nm against 20% piperidine in DMF (v/v) as blank. Resin loading was calculated by using the following equation: resin loading [mmol $g^{-1}$] = (A x 1000) x (m [mg] x 7800 x df)$^{-1}$ with df = dilution factor.

**Fmoc deprotection.** After loading determination, the resin was swelled in dry DCM and Fmoc-protective groups were removed. Therefore, the resin was treated with Fmoc-deprotection solution (20% piperidine in DMF) for 10 minutes. Then, the solution was drained, and the procedure was repeated 3 times. Afterwards, the resin was washed with DMF and DCM (5 x 2 mL each).

**Coupling of Fmoc-protected amino acids and artificial building blocks.** For successive assembly of desired structures, amino acids and artificial building blocks were coupled to the resin in the indicated sequences. Symmetrical branching points were introduced by using Fmoc-*L*-Lys(Fmoc)-OH and asymmetrical branching points were introduced using Fmoc-*L*-Lys(Dde)-OH, for introducing redox cleavage sites, a suitable disulfide-containing, Fmoc-protected building block was used and dimers of Fmoc-amino butanoic acid and Fmoc-amino hexanoic acid served as spacers. For manual coupling, 4 eq of the Fmoc-protected amino acid or artificial building block were dissolved in 500 µL DCM and mixed with 4 eq PyBOP and 4 eq HOBt, both dissolved in 500 µL DMF. After addition of 8 eq of DIPEA, the mixture was added to the resin and incubated for 90 minutes, followed by washing with DMF and DCM (5 x 1 mL each) and removal of Fmoc-group as described above.

**Bocylation.** Prior to removal of Dde protecting group, terminal free amino groups were temporarily protected by Boc protecting group. Therefore, 10 eq of Boc anhydride and 10 eq

of DIPEA were dissolved in DMF and incubated with the resin for 90 min. Completion of Bocylation was confirmed by Kaiser test.

**Dde-deprotection.** Subsequent removal of the Dde protecting group, [*N*-(1-(4,4-dimethyl-2,6-dioxocyclohexylidene)ethyl)], for asymmetrical synthesis was achieved by treatment with 2% hydrazine in DMF (v/v) for 15 cycles of 2 min each. Afterwards, the resin was washed with DMF, 10% DIPEA in DMF (v/v), DMF and DCM (5 x 2 mL each).

**LAF coupling.** For coupling of LAFs, 4 eq of the indicated LAF per deprotected, resin bound amino group and 8 eq of DIPEA were dissolved in DMF, and 4 eq of PyBOP and 4 eq of HOBt were dissolved in DCM. Both solutions were subsequently added to the resin and the reaction time was extended to 36 hours.

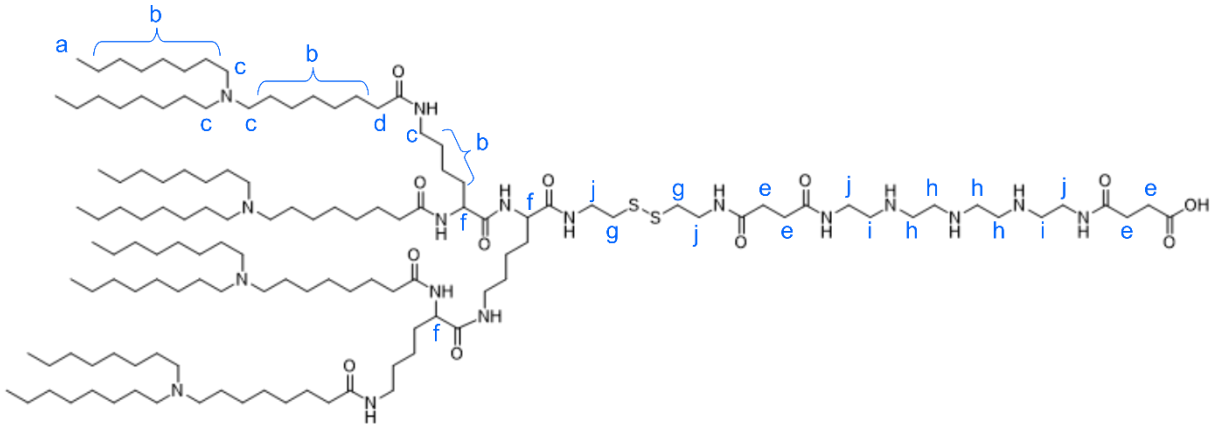
**Kaiser test.** Completion of both, coupling and deprotection steps, was confirmed by performing Kaiser tests. After washing the resin with DCM (5 x 2 mL), a small sample of DCM was transferred into an Eppendorf reaction tube. One drop of each Kaiser test solution was added to the resin: first, 80% (w/v) phenol in EtOH, followed by 5% (w/v) ninhydrin in EtOH and 20 µM potassium cyanide (KCN) in pyridine (1 mL aqueous KCN solution, c = 1 mM in 49 mL pyridine). The tube was incubated under shaking at 500 rpm at 99°C for 4 min. The presence of free amines was indicated by dark blue color of both resin beads and reaction solution. Absence of free amines was indicated by no change of color.

**Cleavage from resin.** Once the desired sequence was obtained, the resin was dried in vacuo prior to cleavage. For cleavage and in situ removal of acid-labile protecting groups, a cleavage cocktail consisting of TFA/TIS/$H_2O$ (95/2.5/2.5 v/v/v) was added to the resin and allowed to react for 45 minutes. The cleavage solution was drained and collected, the resin was washed first with 500 µL of TFA, followed by 1 mL of DCM and the solutions were combined. The collected solutions were dried under nitrogen flow.

**Purification.** The dried pellet was dissolved in EtOH or EtOH/$H_2O$ (1:1, v/v). The clear solutions were dialyzed against EtOH overnight at room temperature, followed by dialysis against $H_2O$ for additional 24 hours at 4°C. For dialysis, Spectra/Por® Dialysis Membranes (Carl Roth, Karlsruhe, Germany), the suitable molecular weight cut-off (MWCO) 1 kDa or 2 kDa were used, depending on the molecular weight of the synthesized sequence. Afterwards, the compounds were diluted with water and lyophilized. Final products were characterized by MALDI-TOF-MS and $^1$H NMR spectroscopy (**Tab. S1**, Supporting information).

## 1.5 Analytical data of LAF-Stp xenopeptides

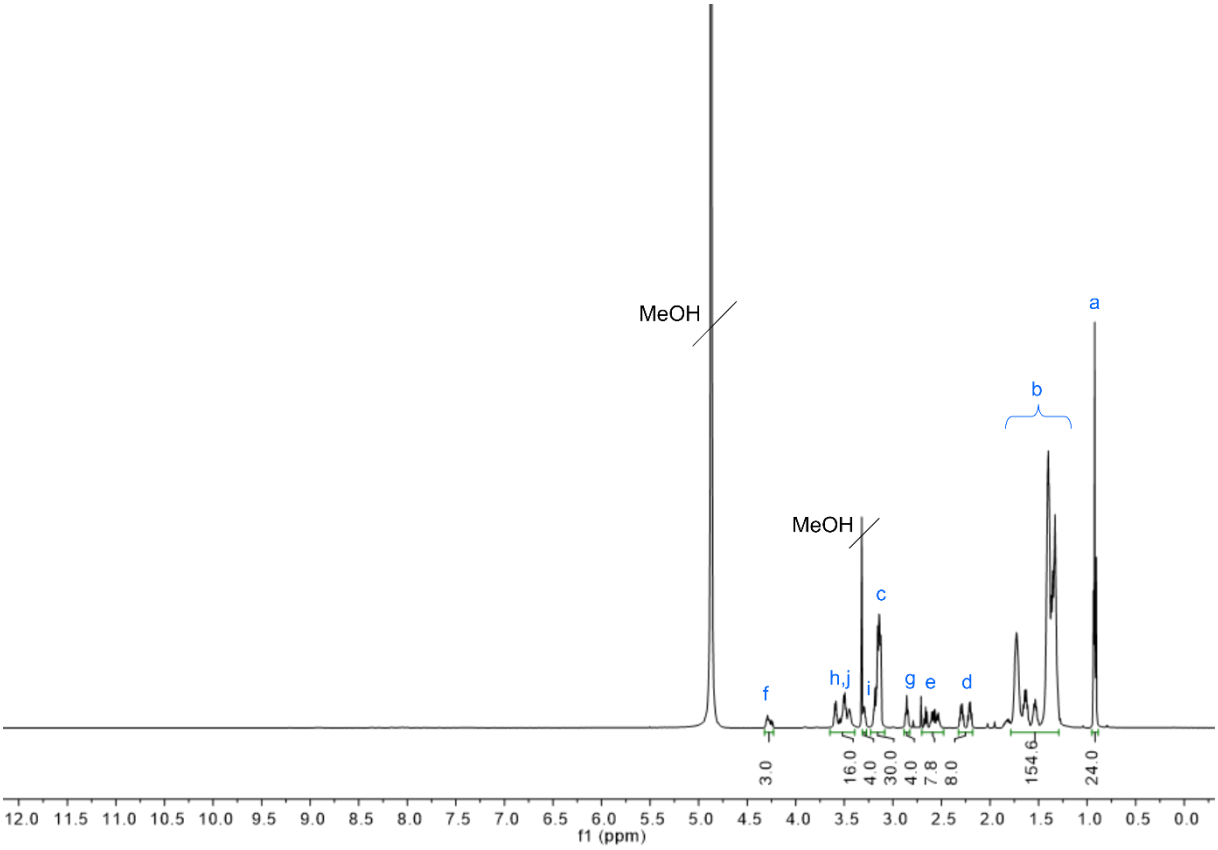
### 1791 (8Oc-B2-1:4-ssbb)

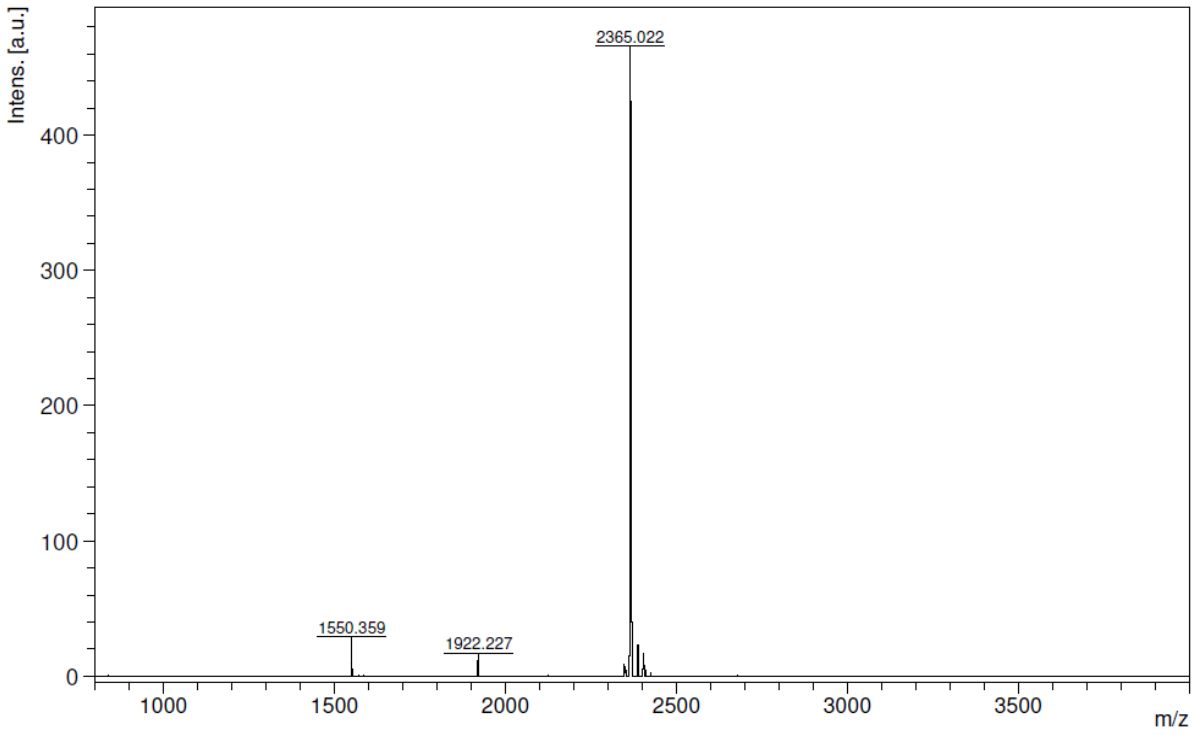


Yield: 26.7 mg (82%)

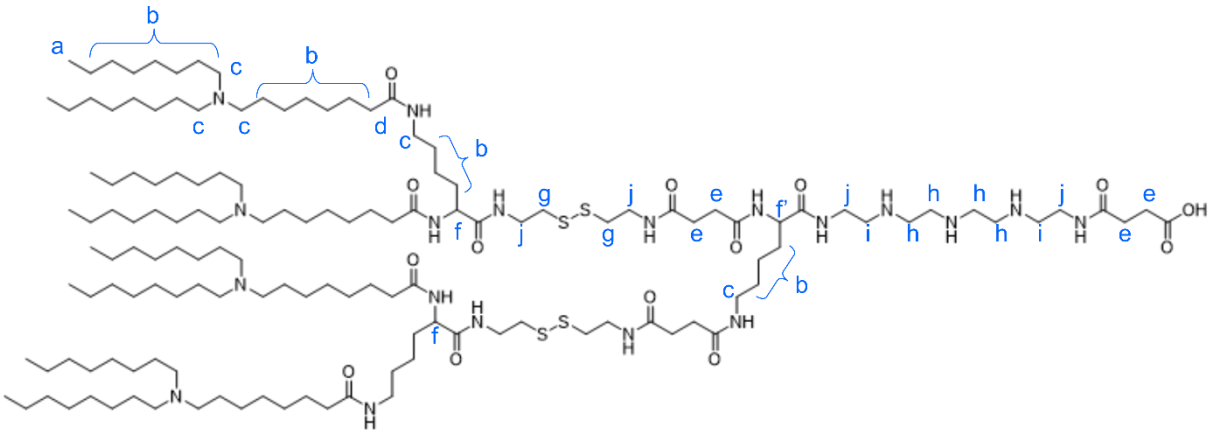
$^1$H NMR (500 MHz, Methanol-$d_4$) $\delta_H$ (ppm) = 4.28 (ddt, $J$ = 19.6, 9.0, 4.0 Hz, 3H, f), 3.64 – 3.39 (m, 16H, h, j), 3.31 – 3.27 (m, 4H, i), 3.22 – 3.07 (m, 30H, c), 2.86 (dd, $J$ = 7.7, 5.3 Hz, 4H, g), 2.70 – 2.48 (m, 8H, e), 2.25 (dtd, $J$ = 42.9, 7.4, 4.7 Hz, 8H, d), 1.80 – 1.26 (m, 154H, b), 0.96 – 0.88 (m, 24H, a).

MALDI-TOF-MS: calculated for $C_{134}H_{266}N_{17}O_{12}S_2$ $[M+H]^+$: 2370.82; found $[M+H]^+$: 2365.02





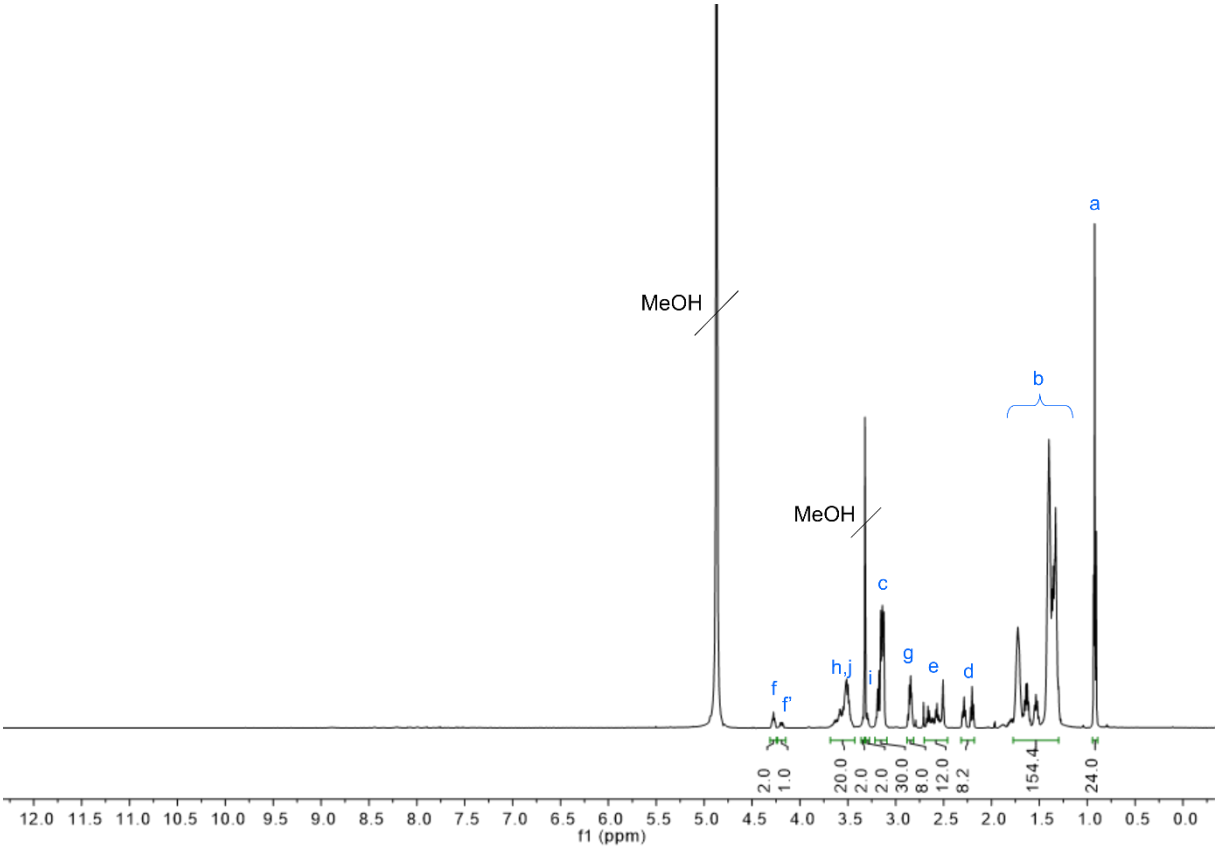

**1792 (8Oc-B2-1:4-ssbb$_2$)**



Yield: 18.6 mg (52%),

$^1$H NMR (500 MHz, Methanol-$d_4$) $\delta_H$ (ppm) = 4.28 (m, 2H, f), 4.19 (dd, J = 9.1, 5.1 Hz, 1H, f'), 3.69 – 3.43 (m, 20H, h, j), 3.32 (m, 4H, i, overlapping signal of solvent residue), 3.22 – 3.09 (m, 30H, c), 2.89 – 2.81 (m, 8H, g), 2.71 – 2.47 (m, 12H, e), 2.32 – 2.13 (m, 8H, d), 1.77 – 1.26 (m, 154H, b), 0.95 – 0.88 (m, 24H, a).

MALDI-TOF-MS: m/z calculated for $C_{142}H_{279}N_{19}O_{14}S_4$ $[M+H]^+$: 2604.07; found $[M+H]^+$: 2598.83


MeOH
MeOH
a
b
c
d
e
f
f'
g
h,j
i
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1.0
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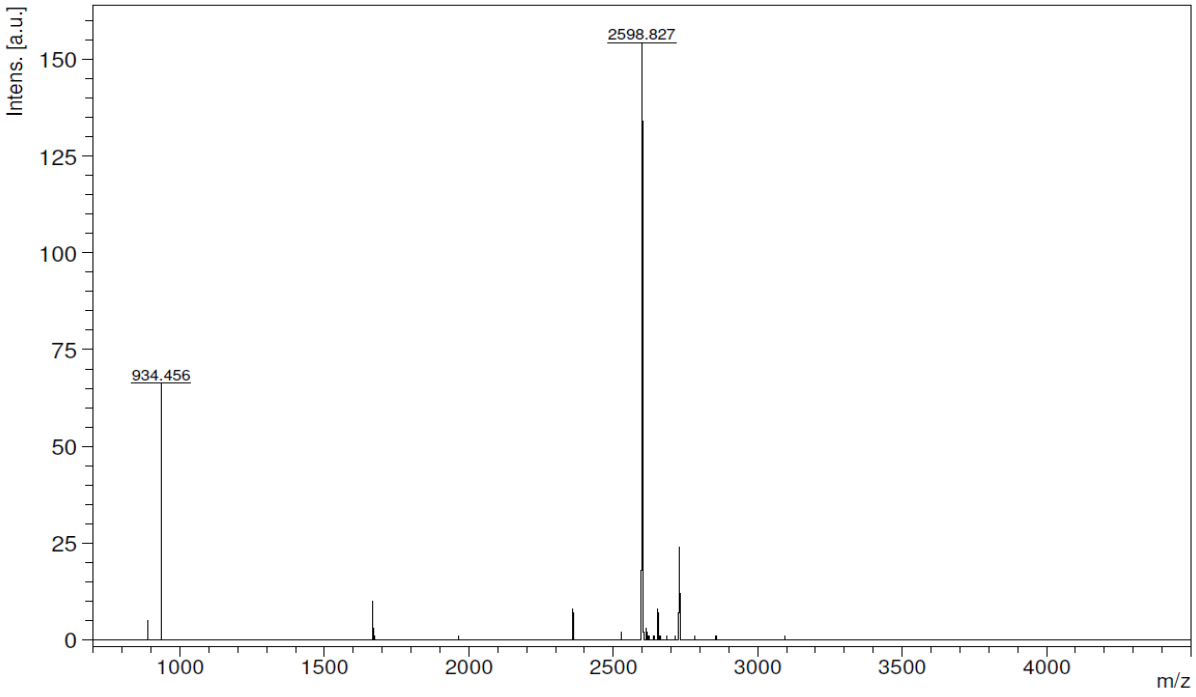
f1 (ppm)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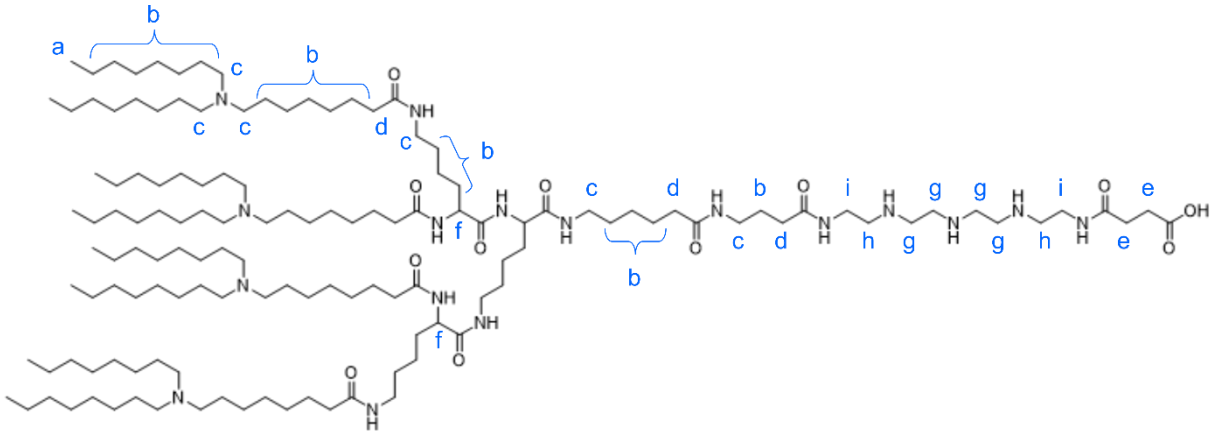
m/z

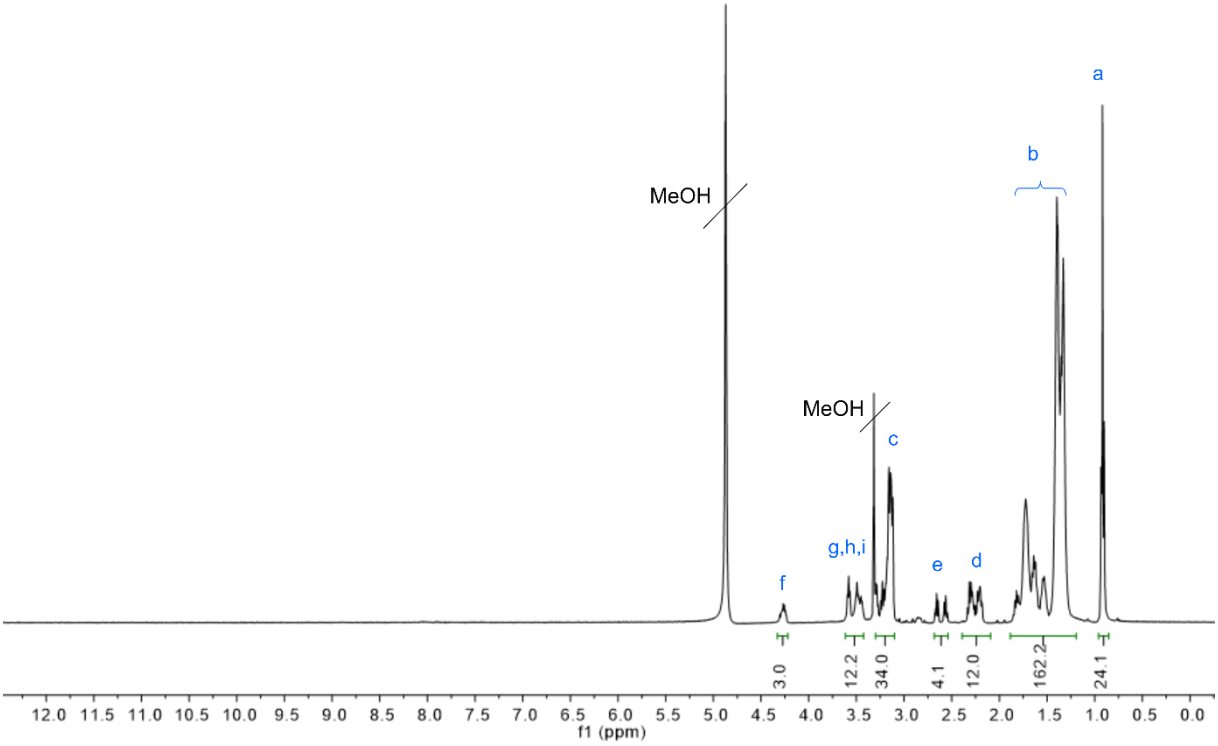
**1793 (8Oc-B2-1:4-spacer)**

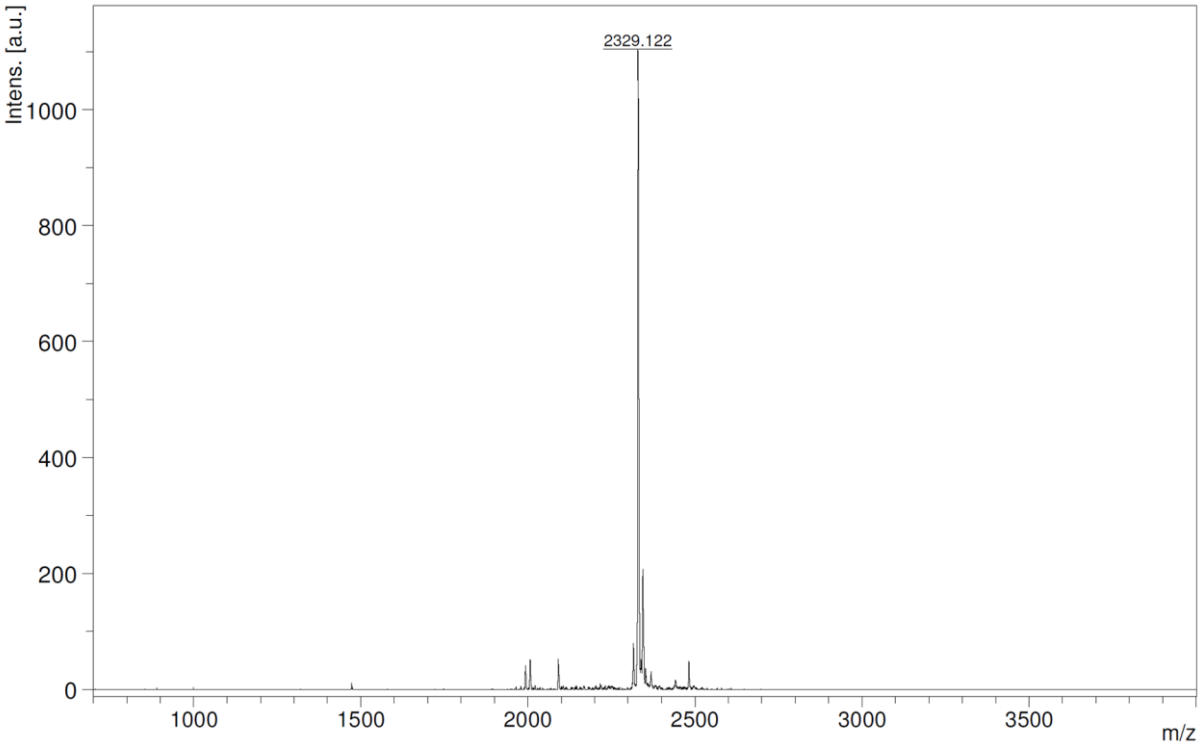


Yield: 14.9 mg, 44%

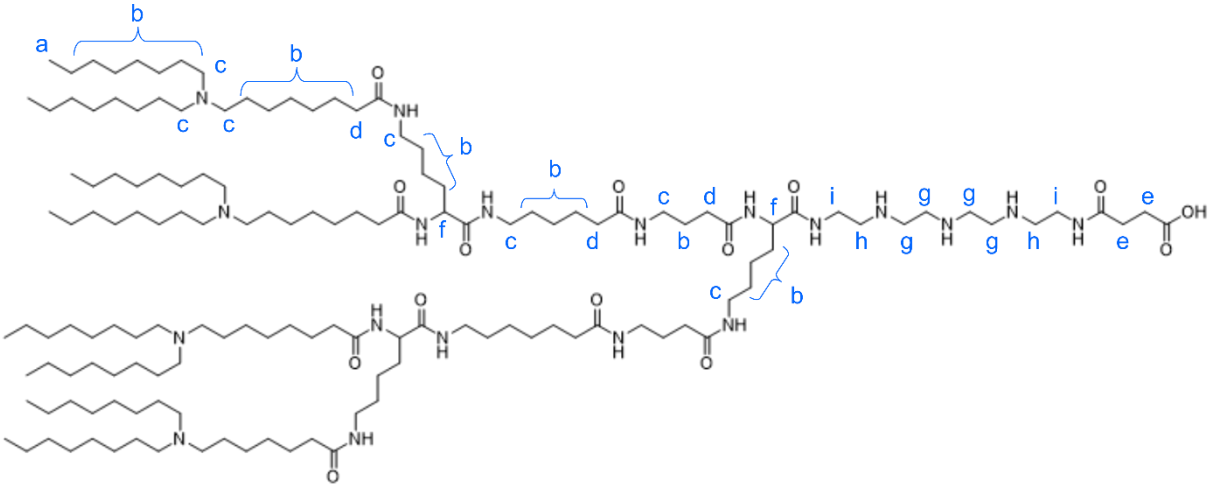
$^{1}$H NMR (400 MHz, Methanol-$d_4$) $\delta_H$ (ppm) = 4.33 – 4.21 (m, 3H, f), 3.63 – 3.38 (m, 12H, g, h, i), 3.31 – 3.09 (m, 34H, c), 2.70 – 2.54 (m, 4H, e), 2.37 – 2.14 (m, 12H, d), 1.88 – 1.19 (m, 162H, b), 0.95 – 0.87 (m, 24H, a).

MALDI-TOF-MS m/z calculated for $C_{136}H_{269}N_{17}O_{12}$ $[M+H]^+$: 2334.11; found $[M+H]^+$: 2329.12





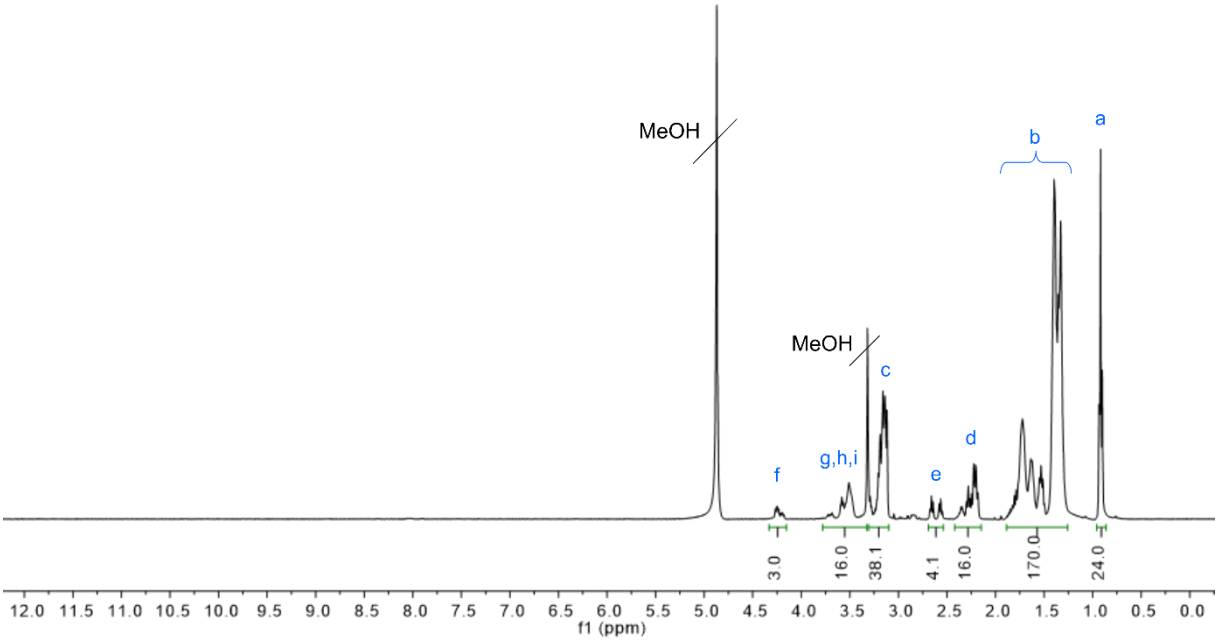

**1794 (8Oc-B2-1:4-spacer$_2$)**

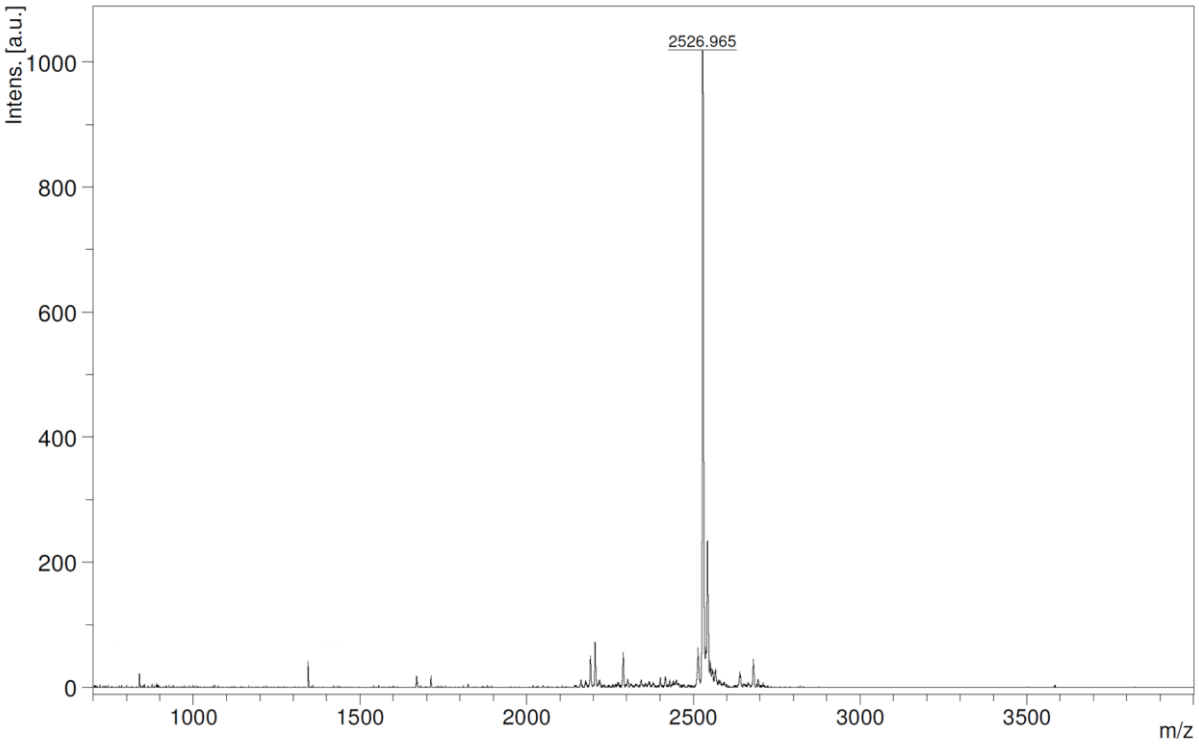


Yield: 19.6 mg, 45%

$^1$H NMR (400 MHz, Methanol-$d_4$) $\delta_H$ (ppm) = 4.31 – 4.17 (m, 3H, f), 3.77 – 3.38 (m, 16H, g, h, i), 3.30 – 3.10 (m, 38H, c), 2.69 – 2.55 (m, 4H, e), 2.41 – 2.15 (m, 16H, d), 1.91 – 1.26 (m, 170H, b), 0.96 – 0.87 (m, 24H, a).

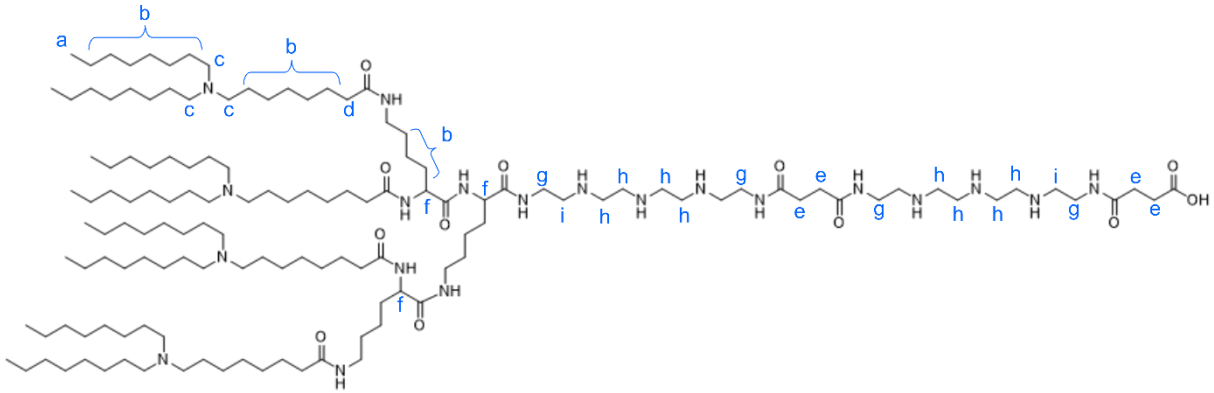
MALDI-TOF-MS m/z calculated for $C_{146}H_{287}N_{19}O_{14}$ $[M+H]^+$ 2532.24; found $[M+H]^+$ 2526.97


MeOH
MeOH
a
b
c
d
e
f
g,h,i
3.0
16.0
38.1
4.1
16.0
170.0
24.0
f1 (ppm)


2526.965
Intens. [a.u.]
m/z

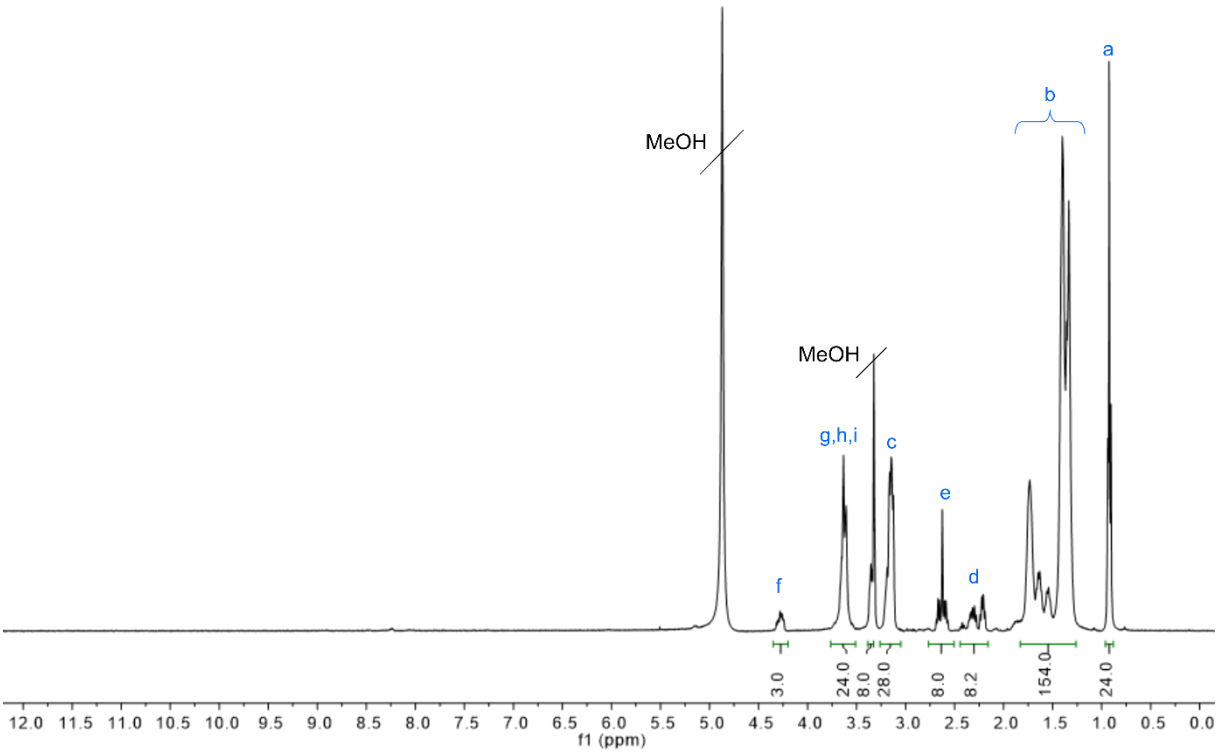
## 8Oc-B2-2:4 library

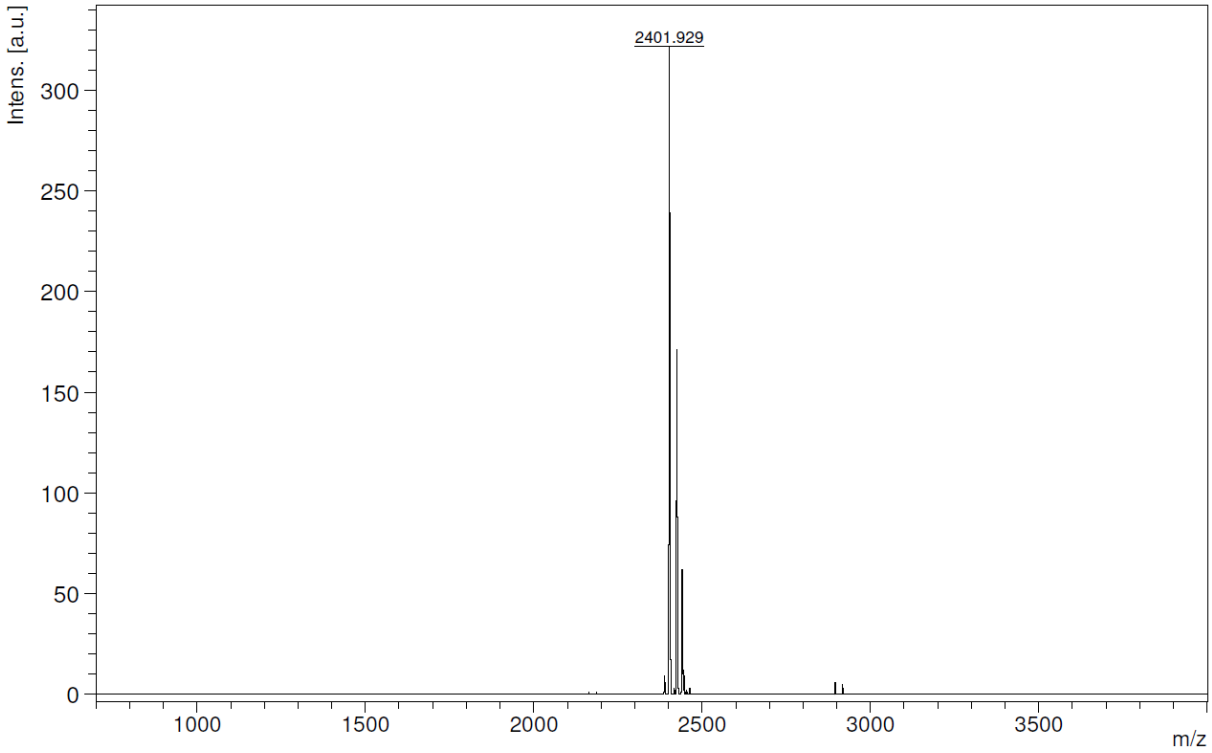
### 1730 (8Oc-B2-2:4)



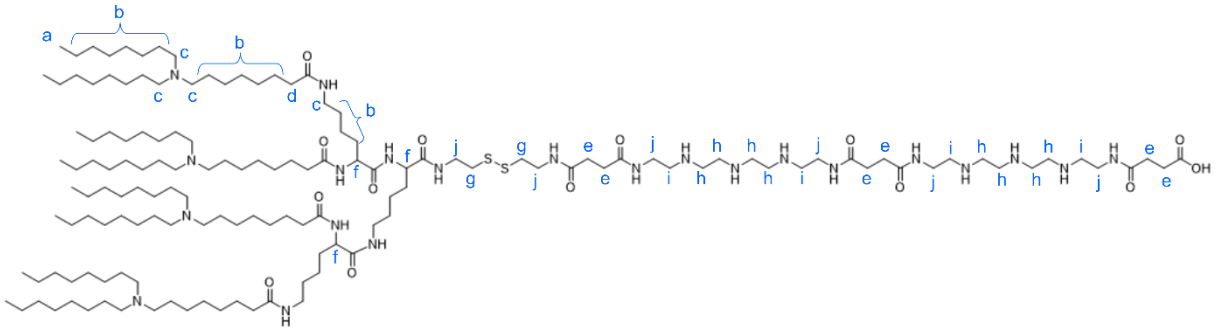
$^{1}$H NMR (400 MHz, Methanol-$d_4$) $\delta_H$ (ppm) = 4.36 – 4.23 (m, 3H, f), 3.64 (td, $J$ = 11.3, 4.6 Hz, 24H, g, h), 3.35 (m, 8H, i), 3.16 (td, $J$ = 11.7, 10.0, 4.5 Hz, 28H, c), 2.72 – 2.57 (m, 8H, e), 2.46 – 2.13 (m, 8H, d), 1.83 – 1.25 (m, 154H, b), 1.00 – 0.86 (m, 24H, a).

MALDI-TOF-MS m/z calculated for $C_{138}H_{276}N_{20}O_{12}$ $[M+H]^+$ 2408.86; found $[M+H]^+$ 2401.93





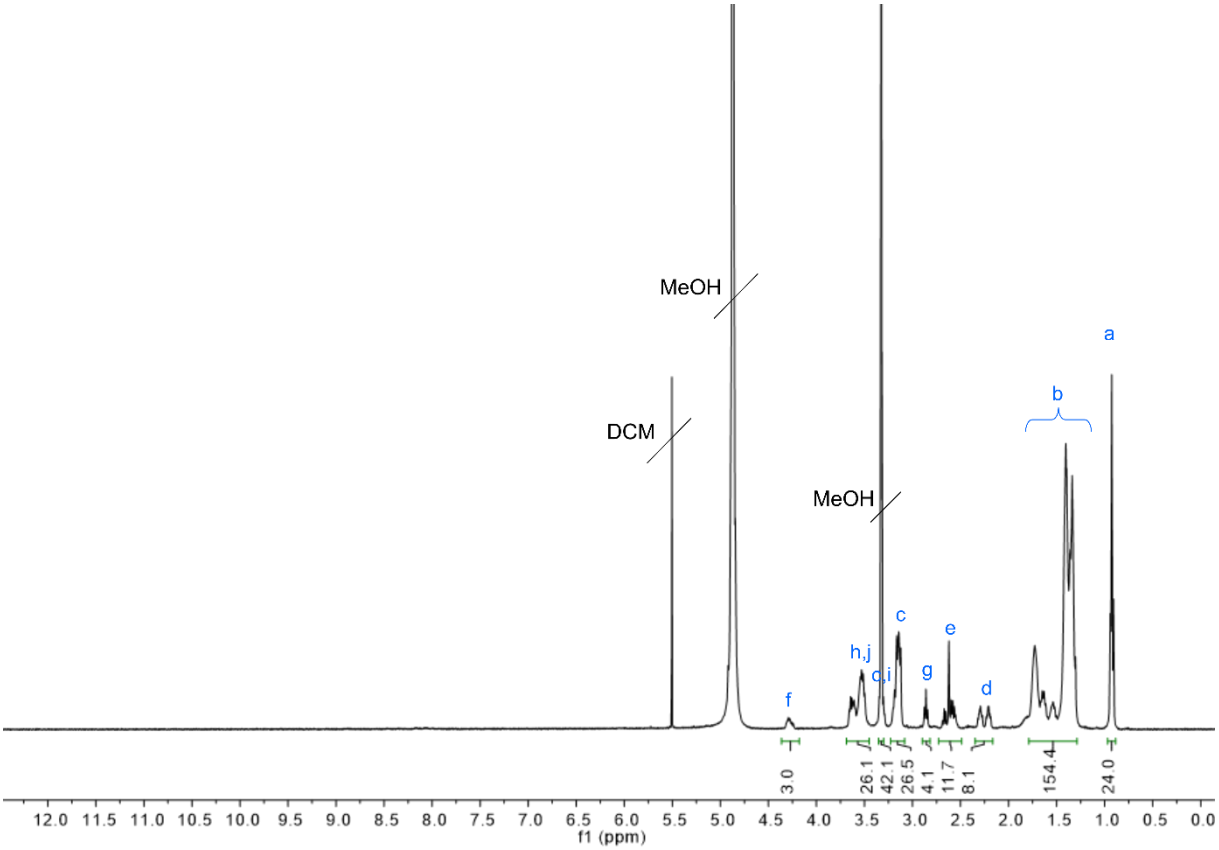

**1823 (8Oc-B2-2:4 ssbb)**

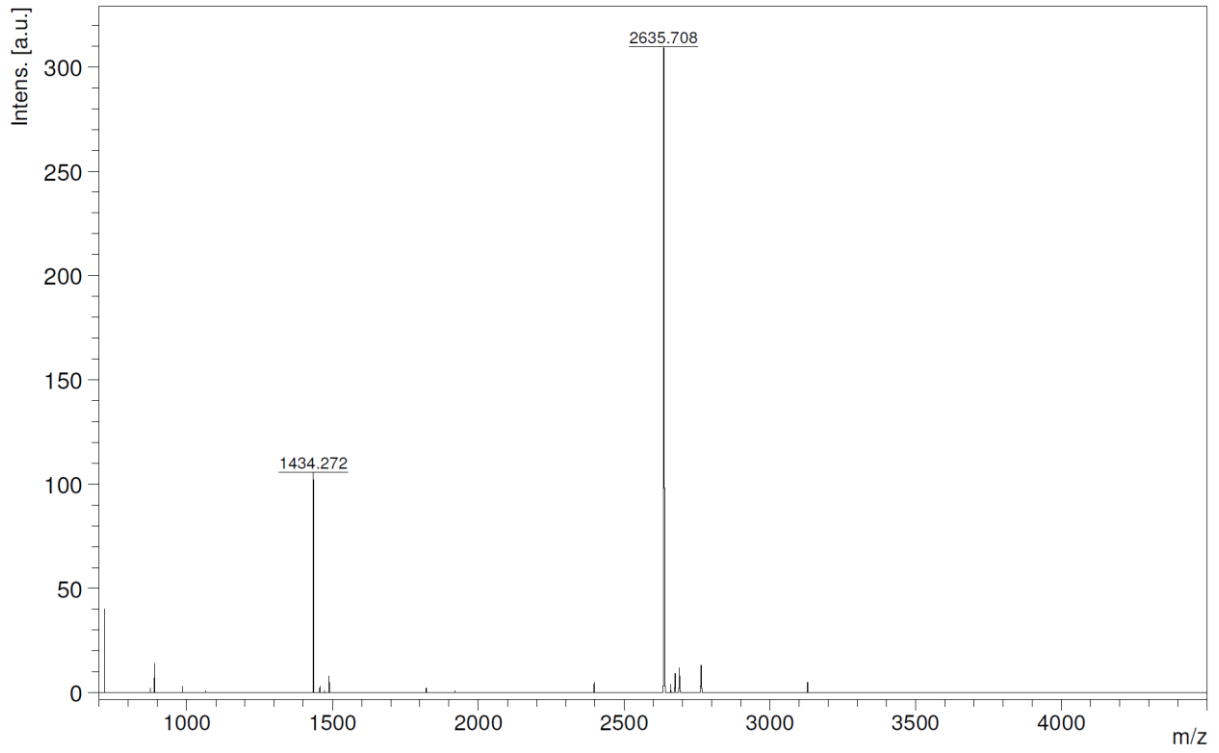


Yield: 15.8 mg, 41%

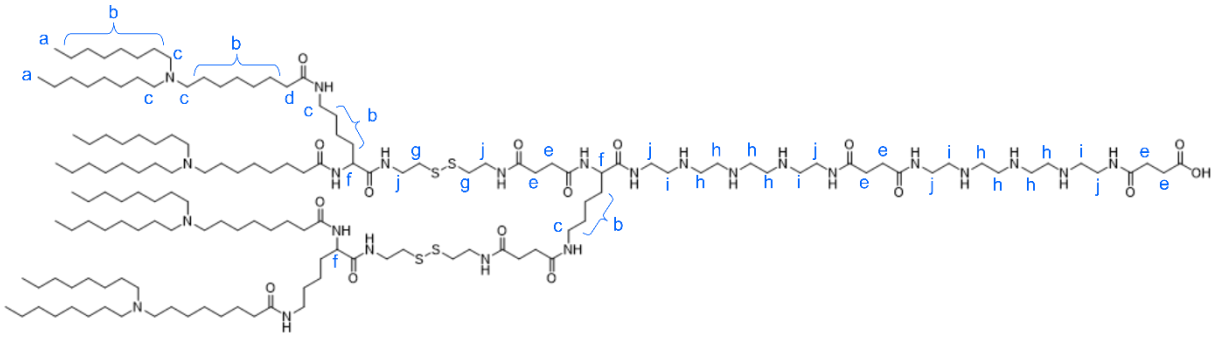
$^{1}$H NMR (400 MHz, Methanol-$d_4$) $\delta_H$ (ppm) =4.34 – 4.22 (m, 3H, f), 3.69 – 3.47 (m, 26H, h,j), 3.33 (m, 12H, c,i, overlapping signal of solvent residue), 3.23 – 3.09 (m, 26H, c), 2.86 (t, J = 6.7 Hz, 4H, g), 2.72 – 2.53 (m, 12H, e), 2.34 – 2.17 (m, 8H, d), 1.80 – 1.28 (m, 154H, b), 0.98 – 0.89 (m, 24H, a).

MALDI-TOF-MS m/z calculated for $C_{146}H_{290}N_{22}O_{14}S_2$ $[M+H]^+$: 2641.22; found $[M+H]^+$: 2635.71


MeOH
DCM
MeOH
a
b
c
e
h,j
g
d
f
3.0
26.1
42.1
26.5
4.1
11.7
8.1
154.4
24.0
f1 (ppm)


Intens. [a.u.]
2635.708
1434.272
m/z

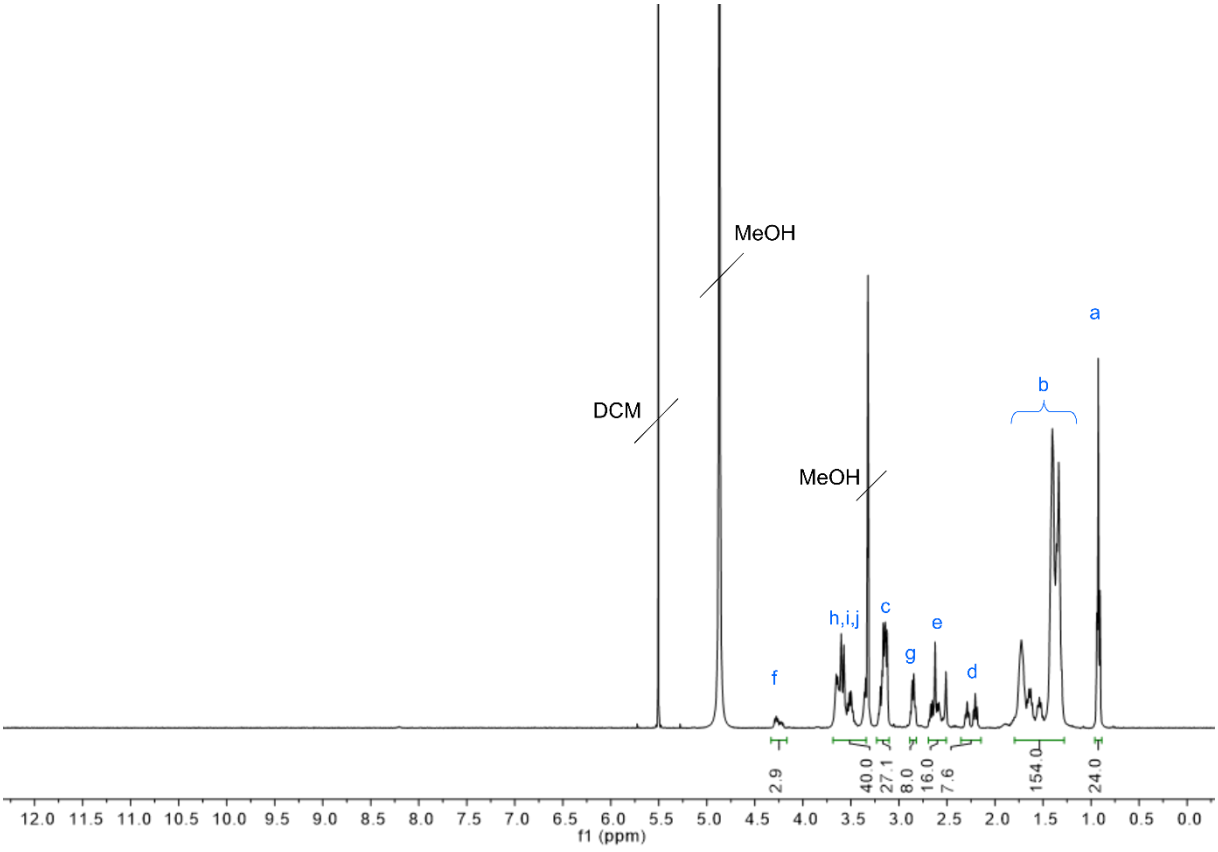
**1824 (8Oc-B2-2:4 -ssbb$_2$)**

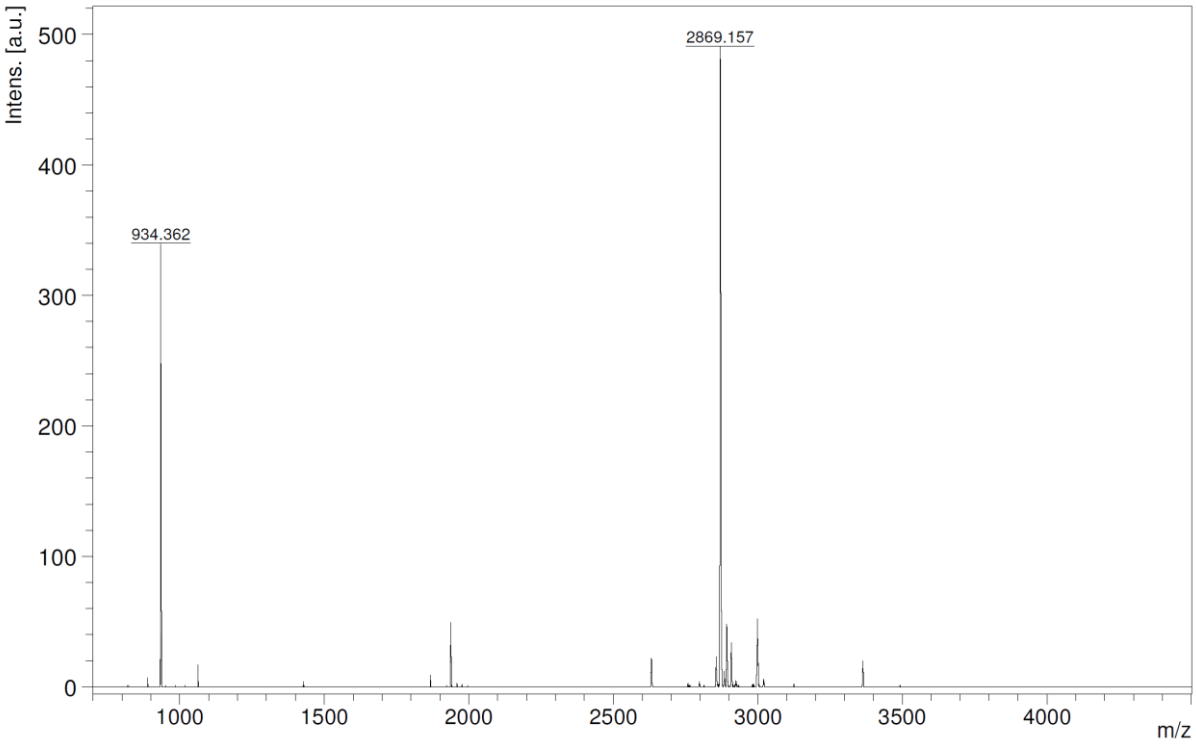


Yield: 25.6 mg, 62%

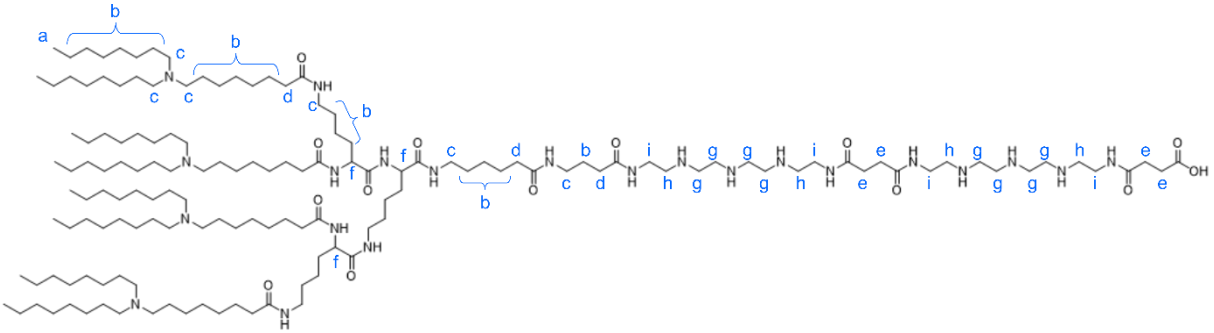
$^1$H NMR (500 MHz, Methanol-$d_4$) $\delta_H$ (ppm) =4.33 – 4.19 (m, 3H, f), 3.70 – 3.34 (m, 40H, h, i, j), 3.24 – 3.09 (m, 27H, c), 2.90 – 2.81 (m, 8H, g), 2.70 – 2.49 (m, 16H, e), 2.34 – 2.15 (m, 8H, d), 1.80 – 1.27 (m, 154H, b), 0.96 – 0.89 (m, 24H, a).

MALDI-TOF-MS m/z calculated for $C_{154}H_{304}N_{24}O_{16}S_4$ $[M+H]^+$: 2874.26; found $[M+H]^+$: 2869.157





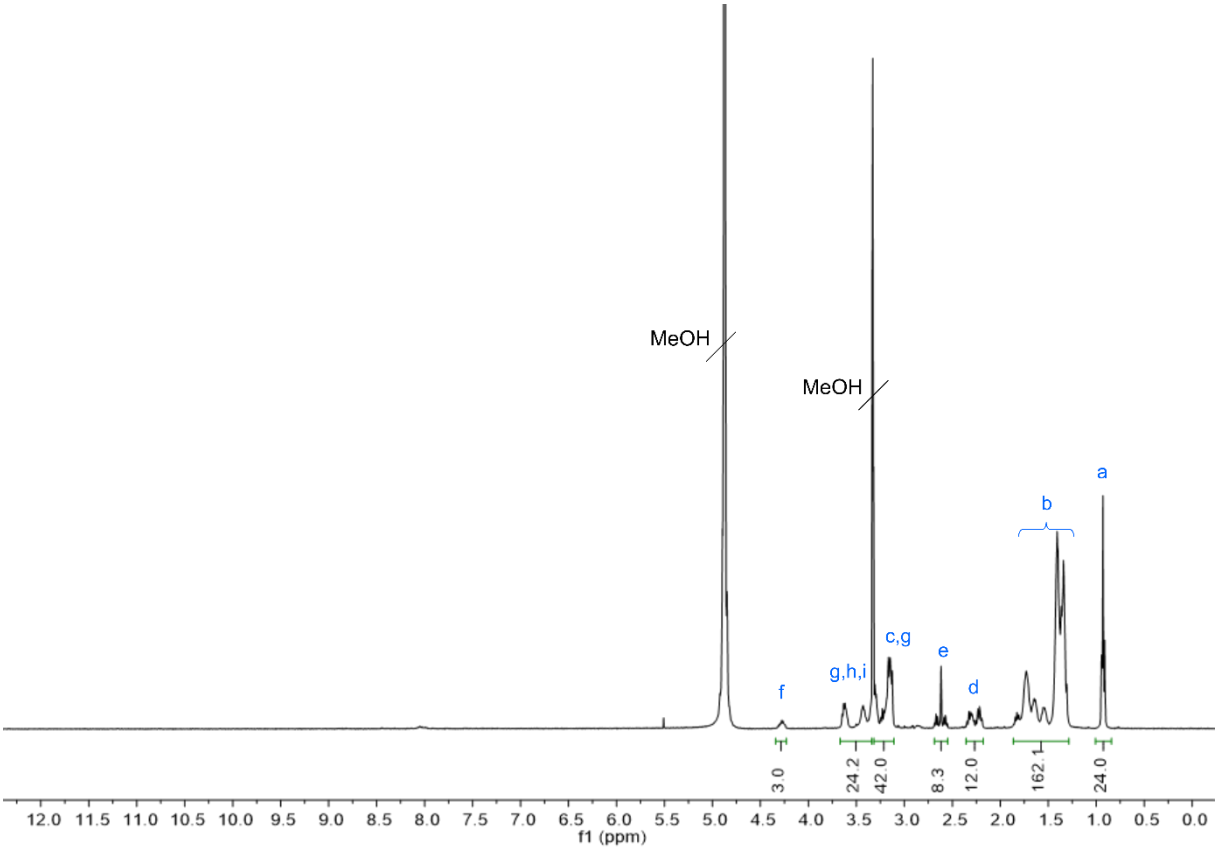

**1825 (8Oc-B2-2:4 -spacer)**

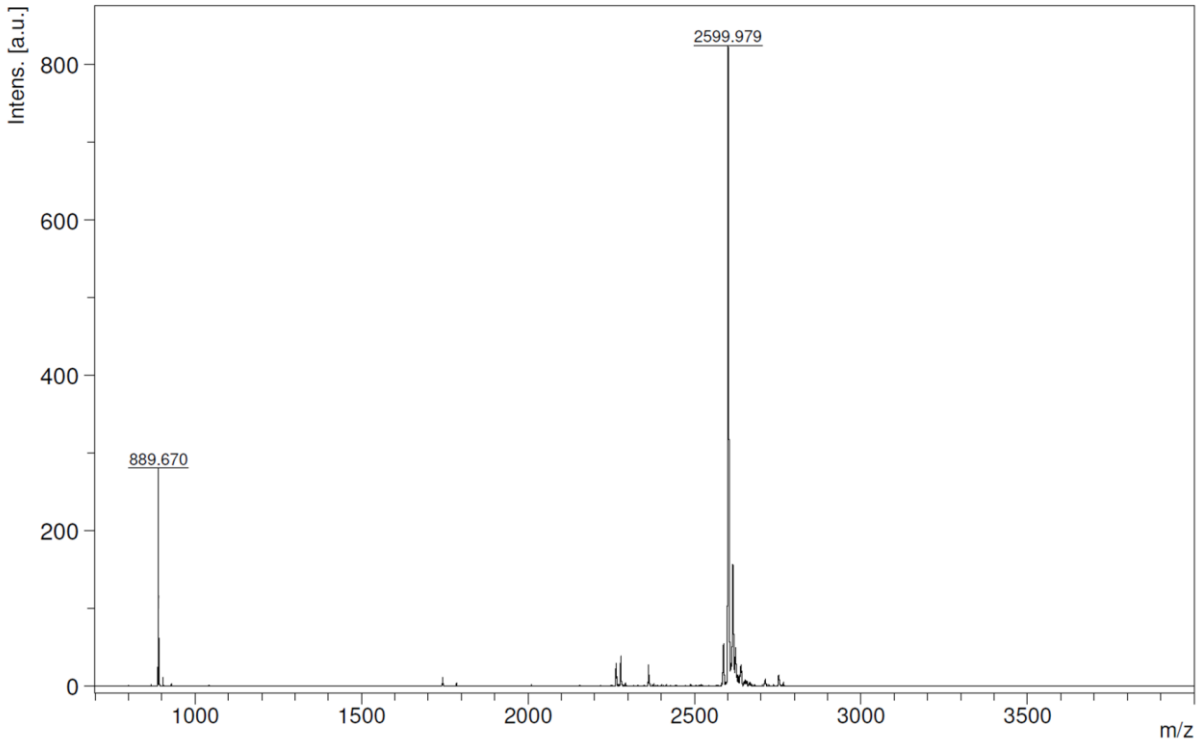


Yield: 28.0 mg, 74%

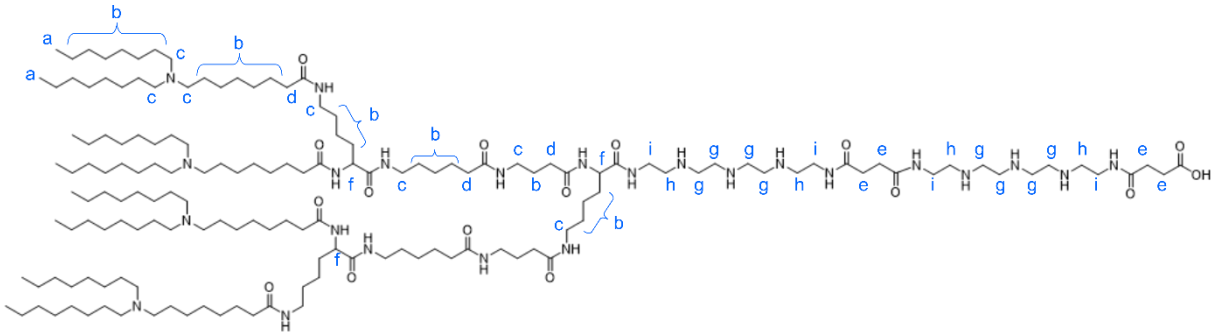
$^1$H NMR (400 MHz, Methanol-$d_4$) $\delta_H$ (ppm) = 4.34 – 4.23 (m, 3H, f), 3.68 – 3.34 (m, 24H, g, h, i, overlapping signal of solvent residue), 3.32 – 3.10 (m, 42H, c, g), 2.70 – 2.54 (m, 8H, e), 2.36 – 2.18 (m, 12H, d), 1.87 – 1.28 (m, 162H, b), 1.00 – 0.87 (m, 24H, a).

MALDI-TOF-MS m/z calculated for $C_{148}H_{294}N_{22}O_{14}$ $[M+H]^+$: 2604.30; found for $[M+H]^+$: 2599.98


MeOH
MeOH
a
b
c,g
e
g,h,i
d
f
3.0
24.2
42.0
8.3
12.0
162.1
24.0
12.0 11.5 11.0 10.5 10.0 9.5 9.0 8.5 8.0 7.5 7.0 6.5 6.0 5.5 5.0 4.5 4.0 3.5 3.0 2.5 2.0 1.5 1.0 0.5 0.0
f1 (ppm)


Intens. [a.u.]
800
600
400
200
0
2599.979
889.670
1000
1500
2000
2500
3000
3500
m/z

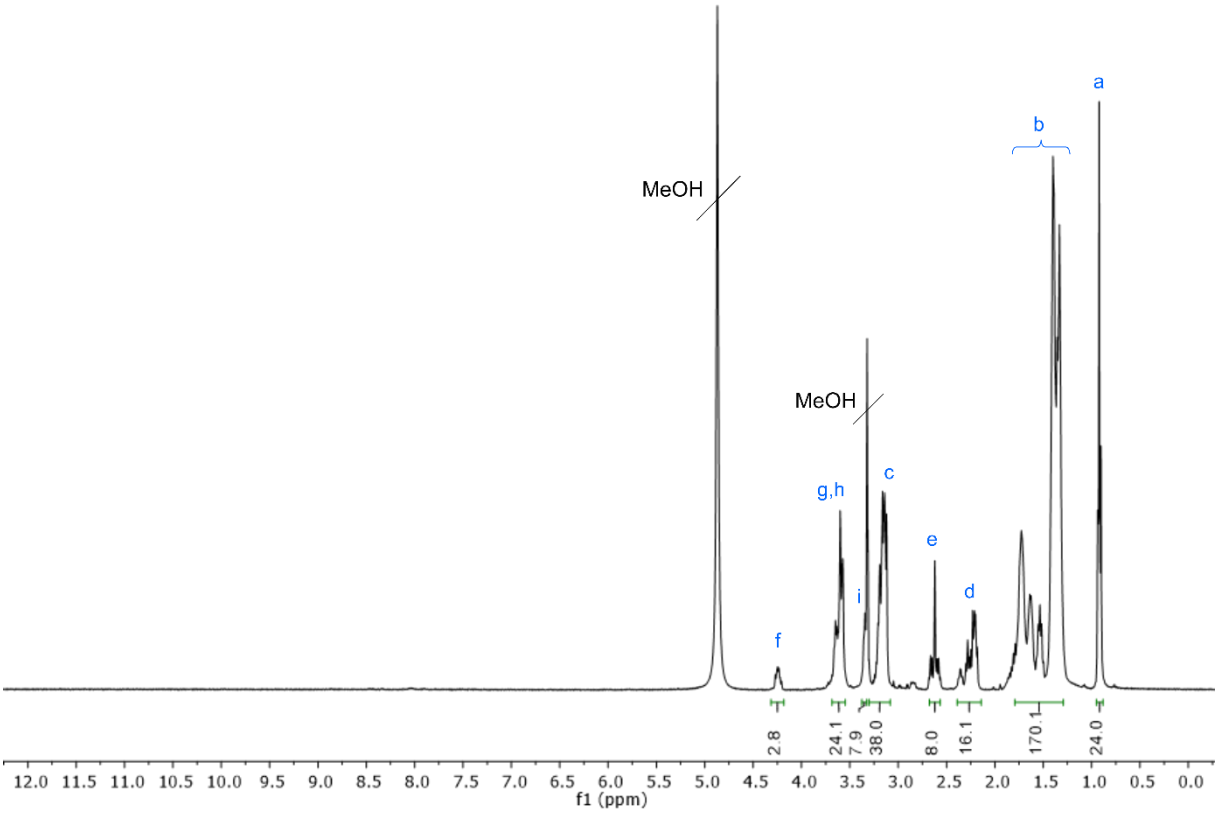
**1826 (8Oc-B2-2:4 -spacer$_2$)**

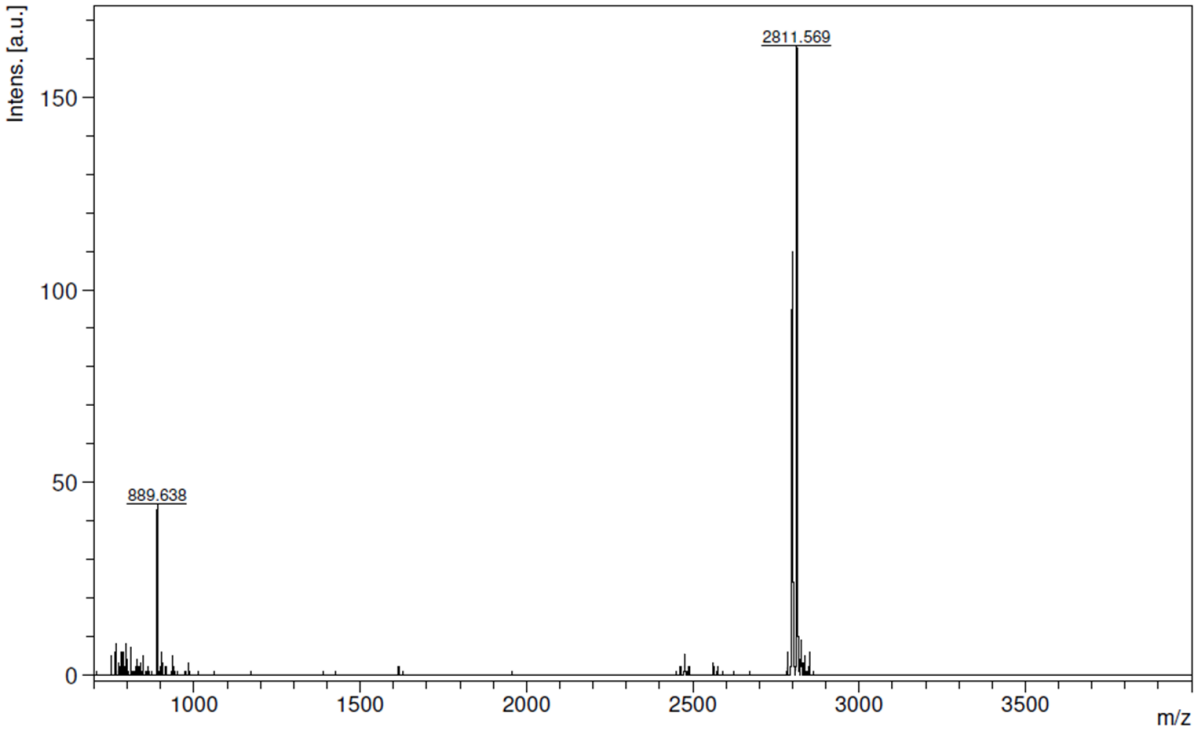


Yield: 14.8 mg, 39%

$^1$H NMR (400 MHz, Methanol-$d_4$) $\delta_H$ (ppm) =4.24 (td, $J$ = 9.9, 5.6 Hz, 3H, f), 3.74 – 3.50 (m, 24H, g, h), 3.38 – 3.33 (m, 8H, i), 3.25 – 3.05 (m, 38H, c), 2.68 – 2.53 (m, 8H, e), 2.41 – 2.15 (m, 16H, d), 1.81 – 1.26 (m, 170H, b), 1.00 – 0.88 (m, 24H, a).

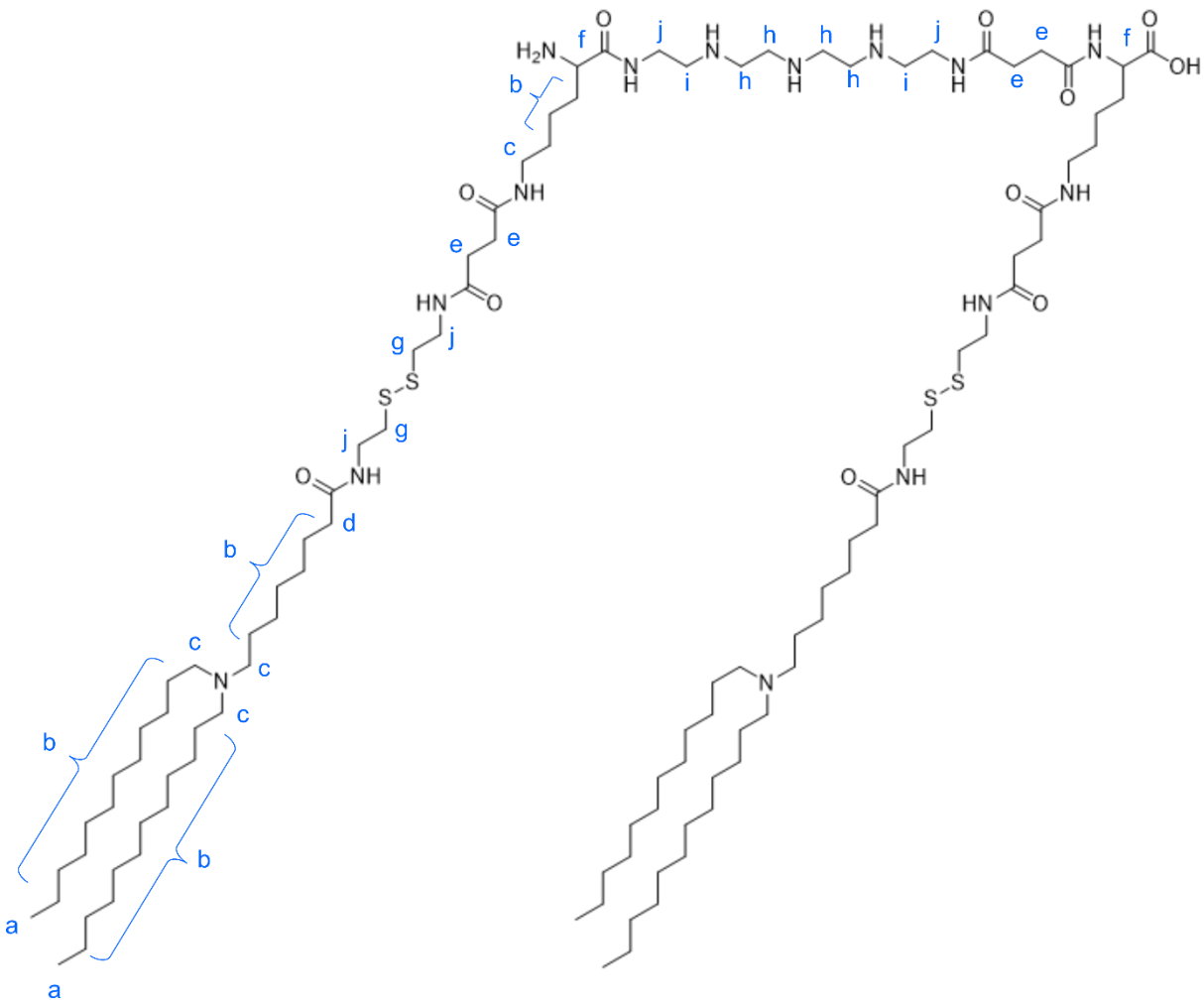
MALDI-TOF-MS m/z calculated for $C_{158}H_{312}N_{24}O_{16}$ $[M+H]^+$: 2802.43; found $[M+H]^+$: 2811.569.




Intens. [a.u.]
150
100
50
0
2811.569
889.638
1000
1500
2000
2500
3000
3500
m/z

**12Oc-U1-1:2 analogs**

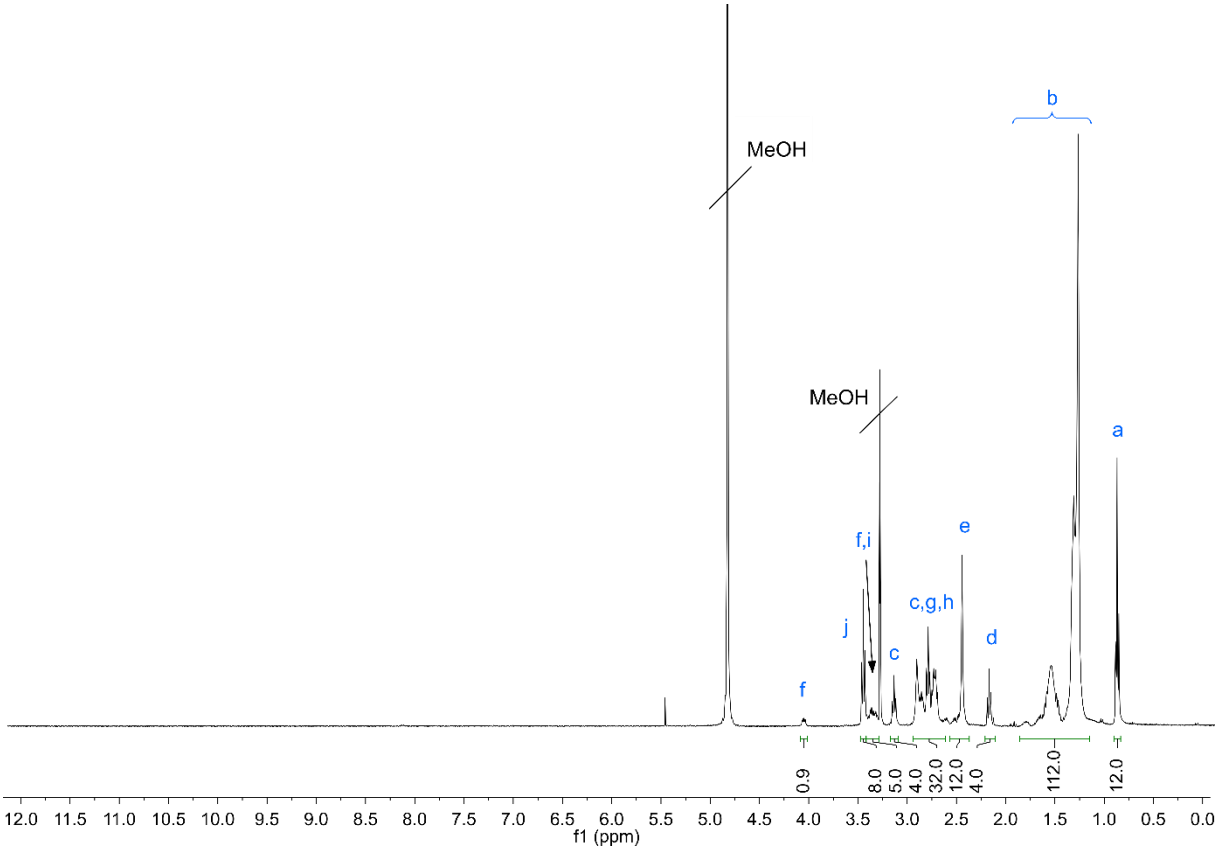
**1821 (12Oc-U1-1:2-ssbb)**

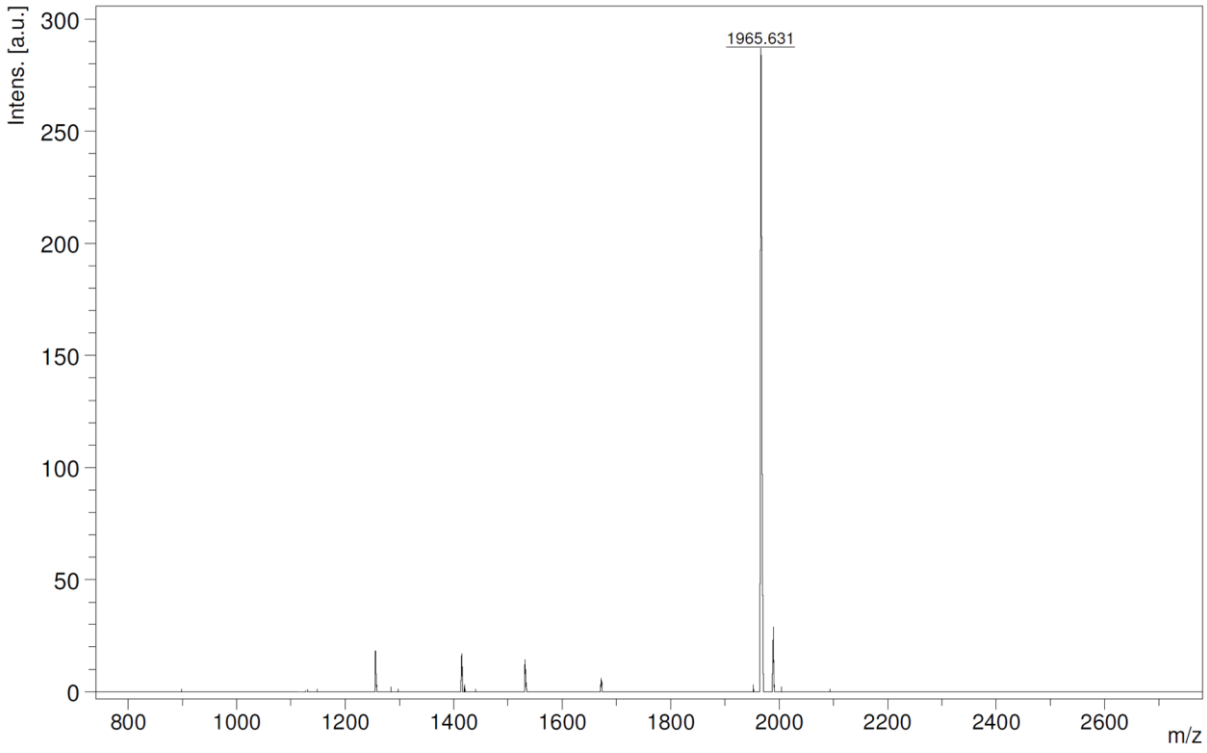


Yield: 17.7 mg, 73%

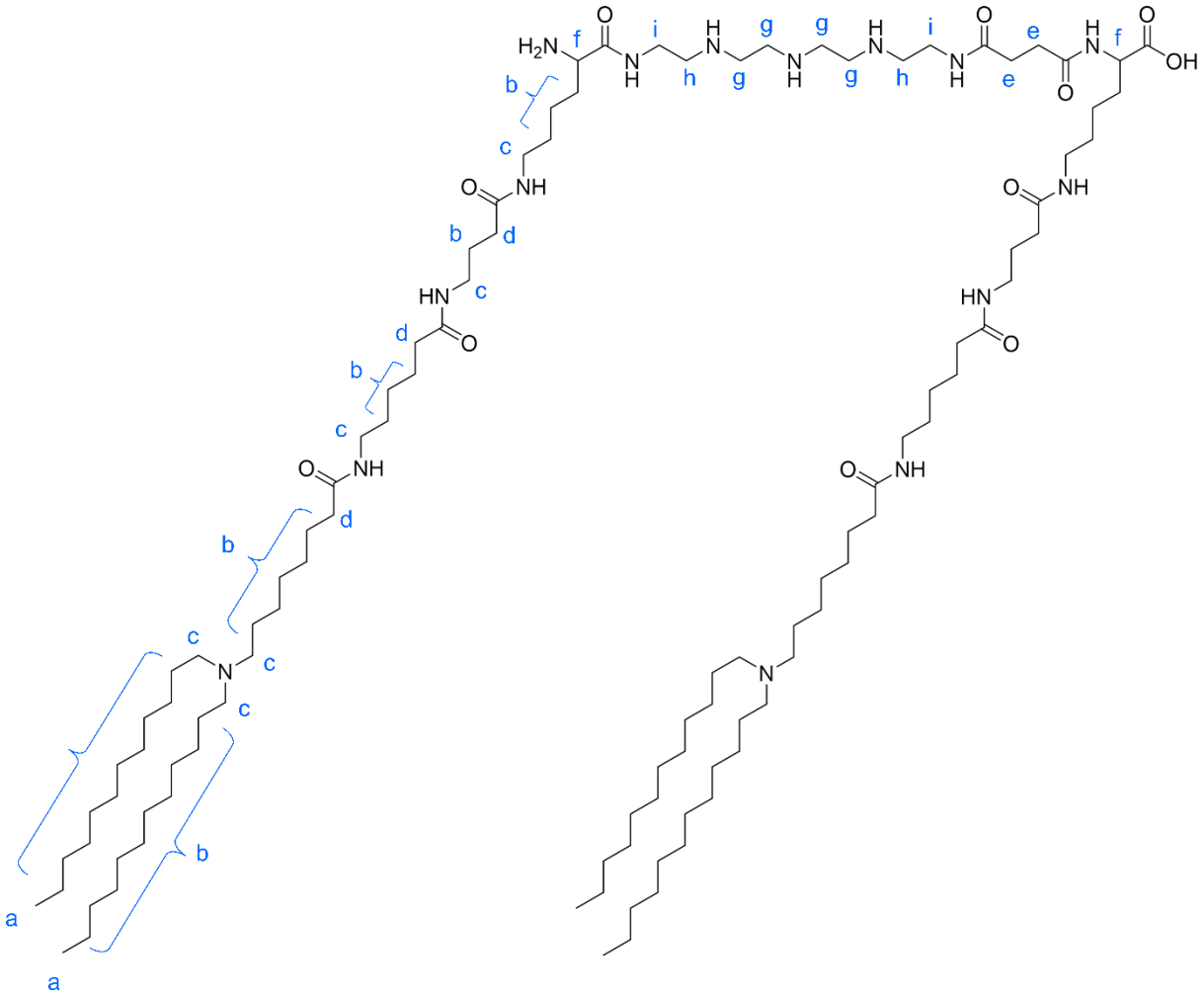
$^{1}$H NMR (400 MHz, Methanol-$d_4$) $\delta_H$ (ppm) = 4.14 – 4.05 (m, 1H, f), 3.49 (t, $J$ = 6.7 Hz, j), 3.44 – 3.33 (m, 5H, f, i), 3.18 (t, $J$ = 6.9 Hz, 4H, c), 3.00 – 2.65 (m, 32H, c, g, h), 2.49 (s, 12H, e), 2.21 (t, $J$ = 7.4 Hz, 4H, d), 1.90 – 1.19 (m, 112H, b), 0.94 – 0.86 (m, 12H, a).

MALDI-TOF-MS m/z calculated for $C_{158}H_{312}N_{24}O_{16}$ $[M+H]^+$: 1968.483; found $[M+H]^+$: 1965.631


MeOH
MeOH
b
a
e
f,i
c,g,h
j
c
d
f
0.9
8.0
5.0
4.0
32.0
12.0
4.0
112.0
12.0
f1 (ppm)


1965.631
Intens. [a.u.]
m/z

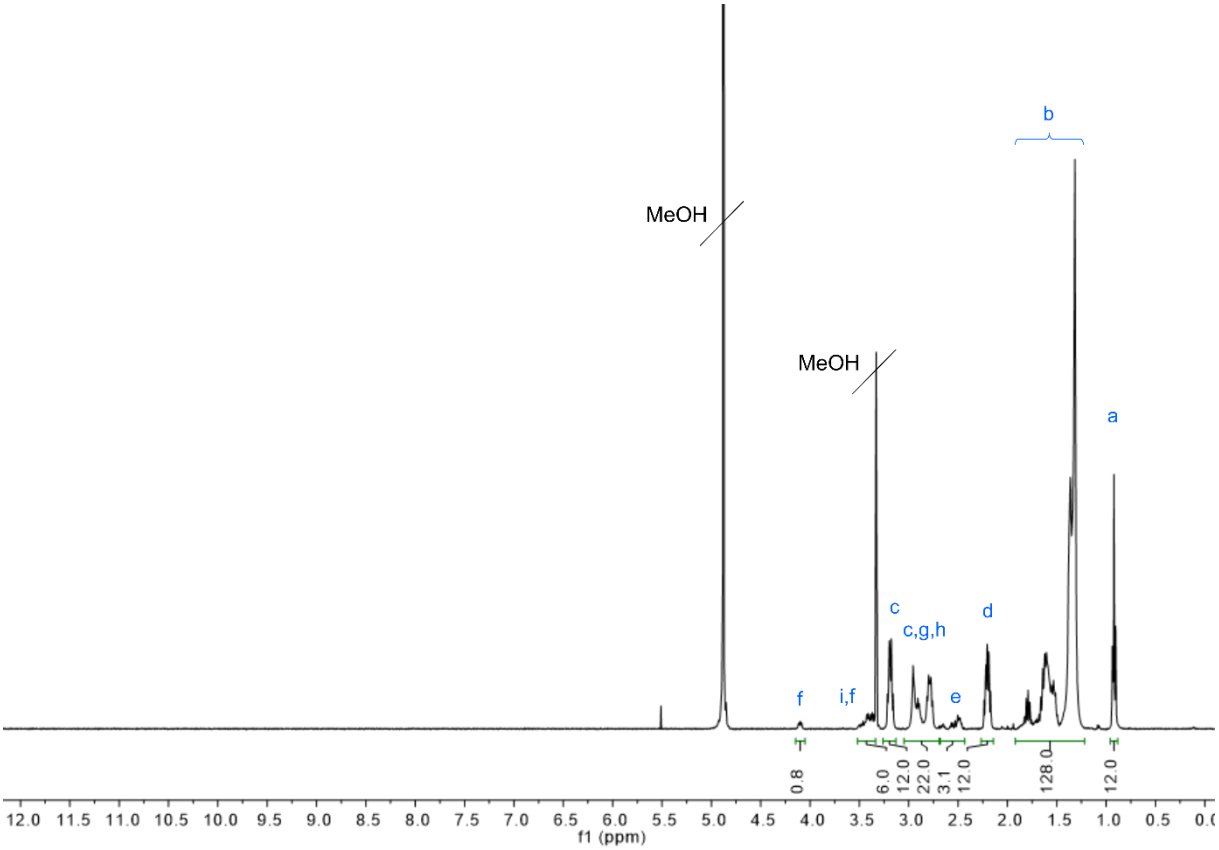
**1822 (12Oc-U1-1:2-spacer)**

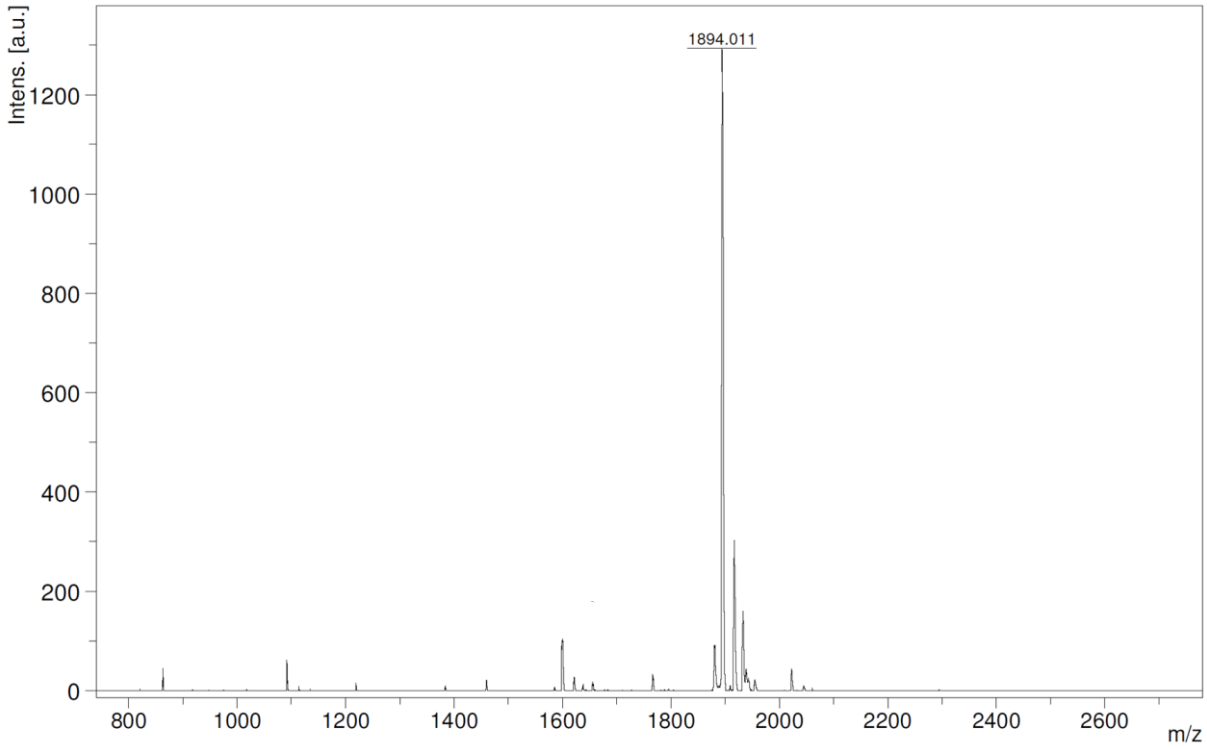


Yield: 15.3 mg, 52%

$^{1}$H NMR (400 MHz, Methanol-$d_4$) $\delta_H$ (ppm) = 4.10 (dd, $J$ = 8.6, 4.7 Hz, 1H, f), 3.52 – 3.33 (m, 5H, f, i), 3.24 – 3.13 (m, 12H, c), 3.03 – 2.70 (m, 22H, g, h, c), 2.68 – 2.42 (m, 4H, e), 2.26 – 2.16 (m, 12H, d), 1.92 – 1.23 (m, 128H, b), 0.97 – 0.87 (m, 12H, a).

MALDI-TOF-MS m/z calculated for $C_{108}H_{213}N_{15}O_{11}$ $[M+H]^+$: 1896.66; found $[M+H]^+$: 1894.011


MeOH
MeOH
b
a
c
c,g,h
d
f
i,f
e
0.8
6.0
12.0
22.0
3.1
12.0
128.0
12.0
f1 (ppm)


1894.011
Intens. [a.u.]
m/z

# 2 Supporting Tables

**Table S1:** Summarizing table with mass data for all LAF-Stp oligomers.

| ID | Sequence (N to C) | EtOH/ $H_2O$ (v/v)[(a)] | Formula | Calculated mass | Found mass (MALDI-TOF-MS) |
|---|---|---|---|---|---|
| **Bundles 8Oc-B2-1:4** | | | | | |
| **1621** | $[K(8Oc)_2]_2$-K-Stp | 9:1* | $C_{126}H_{251}N_{15}O_{10}$ | 2136.43 | 2133.46 [4] |
| **1791** | $[K(8Oc)_2]_2$-K-ssbb-Stp | 9:1 | $C_{134}H_{265}N_{17}O_{12}S_2$ | 2369.01 | 2365.02 |
| **1792** | $[K(8Oc)_2$-ssbb$]_2$-K-Stp | 9:1 | $C_{142}H_{279}N_{19}O_{14}S_4$ | 2603.06 | 2598.83 |
| **1793** | $[K(8Oc)_2]_2$-K-$C_6$-$C_4$-Stp | 9:1 | $C_{136}H_{269}N_{17}O_{12}$ | 2333.10 | 2329.12 |
| **1794** | $[K(8Oc)_2$-$C_6$-$C_4]_2$-K-Stp | 9:1 | $C_{146}H_{287}N_{19}O_{14}$ | 2531.23 | 2526.97 |
| **Bundles 8Oc-B2-2:4** | | | | | |
| **1730** | $[K(8Oc)_2]_2$-K-$Stp_2$ | 9:1 | $C_{138}H_{276}N_{20}O_{12}$ | 2407.85 | 2401.93 |
| **1823** | $[K(8Oc)_2]_2$-K-ssbb-$Stp_2$ | 9:1 | $C_{146}H_{290}N_{22}O_{14}S_2$ | 2640.21 | 2635.71 |
| **1824** | $[K(8Oc)_2$-ssbb$]_2$-K-$Stp_2$ | 9:1 | $C_{154}H_{304}N_{24}O_{16}S_4$ | 2874.26 | 2869.16 |
| **1825** | $[K(8Oc)_2]_2$-K-$C_6$-$C_4$-$Stp_2$ | 9:1 | $C_{148}H_{294}N_{22}O_{14}$ | 2604.03 | 2599.98 |
| **1826** | $[K(8Oc)_2$-$C_6$-$C_4]_2$-K-$Stp_2$ | 9:1 | $C_{158}H_{312}N_{24}O_{16}$ | 2802.43 | 2811.57 |
| **U-shapes 12Oc-U1-1:2** | | | | | |
| **1611** | K(12Oc)-Stp-K(12Oc) | 1:1 | $C_{88}H_{177}N_{11}O_7$ | 1501.42 | 1499.15 |
| **1821** | K(ssbb-12Oc)-Stp-K(ssbb-12Oc) | 10:0 | $C_{104}H_{205}N_{15}O_{11}S_4$ | 1968.48 | 1965.63 |
| **1822** | K($C_4$-$C_6$-12Oc)-Stp-K($C_4$-$C_6$-12Oc) | 10:0 | $C_{108}H_{213}N_{15}O_{11}$ | 1896.66 | 1894.01 |

[(a)] solvent for dissolution; abbreviations: ID, identification number; EtOH, ethanol, MALDI-TOF-MS, matrix-assisted laser desorption ionization – time of flight – mass spectrometry; 8Oc, LAF based on 8-aminooctanoic acid and two octyl chains; 12Oc, LAF based on 8-aminooctanoic acid and two dodecyl chains, K, lysine; Stp, succinoyl tetraethylene pentamine; ssbb, disulfide building block; $C_6$, spacer building block based on 6-aminohexanoic acid; $C_4$, spacer building block based on 4-aminobutyric acid. *dissolved in ethanol/$HCl_{aq}$

**Table S2:** Summarizing table with mass data for disulfide-containing LAF- Stp oligomers after incubation with 10 mM glutathione (GSH) for 90 minutes at 37°C.

| Oligomer | Fragment structures (N → C) | calculated $[M+H]^+$ | found | comment |
|---|---|---|---|---|
| **8Oc-B2-1:4** | | | | |
| **1791** | $[K(8Oc)_2]_2$-K-ssbb-Stp | 2370.0 | 2365.9 | $[M+H]^+$ |
| | $[K(8Oc)_2]_2$-K-SH | 1922.8 | 1915.2 | $[M+H]^+$ |
| | SH-Stp | 449.3 | n.d. | outside detection range |
| **1792** | $[K(8Oc)_2$-ssbb$]_2$-K-Stp | 2604.1 | 2598.9 | $[M+H]^+$ |
| | $K(8Oc)_2$- SH | 936.9 | 934.5 | $[M+H]^+$ |
| | $[K(8Oc)_2$-ssbb]-K(SH)-Stp | 1670.2 | 1667.0 | $[M+H]^+$ |
| | Stp-K-$(SH)_2$ | 736.4 | n.d. | outside detection range |
| **8Oc-B2-2:4** | | | | |
| **1823** | $[K(8Oc)_2]_2$-K-ssbb-$Stp_2$ | 2641.2 | 2635.9 | $[M+H]^+$ |
| | $[K(8Oc)_2]_2$-K-SH | 1923.8 | 1915.2 | $[M+H]^+$ |
| | SH-$Stp_2$ | 720.5 | 1435.9 | $[2M+H]^+$ |
| **1824** | $[K(8Oc)_2$-ssbb$]_2$-K-$Stp_2$ | 2875.3 | 2871.2 | $[M+H]^+$ |
| | $K(8Oc)_2$-SH | 936.9 | 934.5 | $[M+H]^+$ |
| | $[K(8Oc)_2$-ssbb]-K(SH)-$Stp_2$ | 1941.4 | 1937.9 | $[M+H]^+$ |
| | (SH)-K-$Stp_2$ | 1007.6 | n.d. | not detected |
| **12Oc-U1-1:2** | | | | |
| **1821** | K-ε(ssbb-12Oc)-Stp-K-ε(ssbb-12Oc) | 1969.5 | 1965.5 | $[M+H]^+$ |
| | 12Oc-SH | 555.5 | 1105.2 | $[2M+H]^+$ |
| | K-ε(ssbb-12Oc)-Stp-K-ε(SH) | 1417.0 | 1413.6 | $[M+H]^+$ |
| | K-ε(SH)-Stp-K-ε(SH) | 864.5 | 858.4, 1718.1 | $[M+H]^+$, $[2M+H]^+$ |

K, lysine; Stp, succinoyl tetraethylene pentamine; ssbb, disulfide building block; 8Oc, LAF based on 8-aminooctanoic acid and two octyl chains; 12Oc, LAF based on 8-aminooctanoic acid and two dodecyl chains; n.d., not detected.

# 3 Supporting Figures

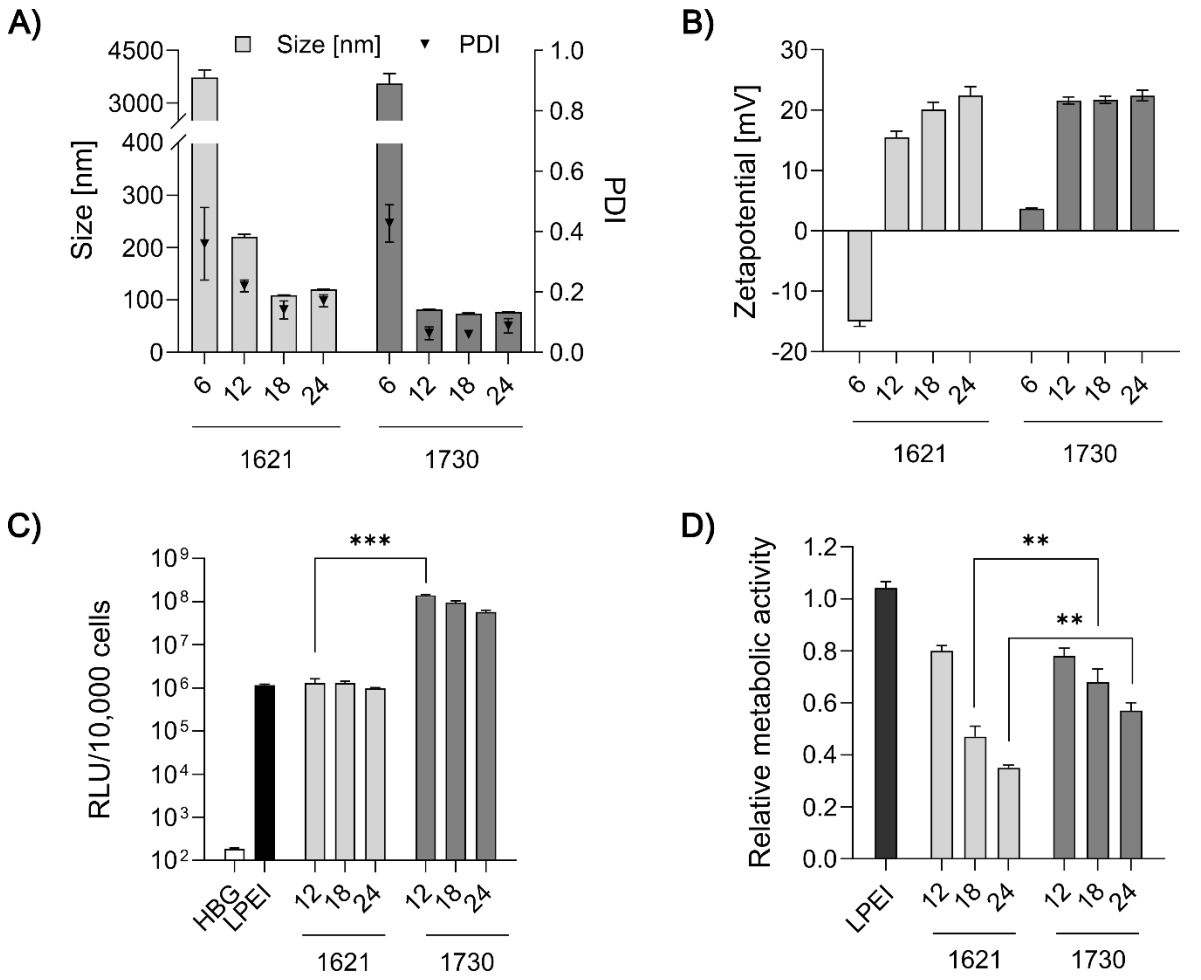



**Figure S1.** Evaluation of pDNA polyplexes after chemical evolution from 8Oc-B2-1:4 (1621) to 8Oc-B2-2:4 (1730). A) and B) Evaluation of different N/P ratios on physico-chemical properties of pDNA polyplexes by DLS and ELS measurements. C) and D) *In vitro* evaluation on N2a cells (n = 3, mean + SD). Cellular transfection efficiency was determined by luciferase gene expression assay (C). Cell viability at 24 hours after transfection was determined by CellTiter Glo® Assay in relation to control wells treated with HBG (D).

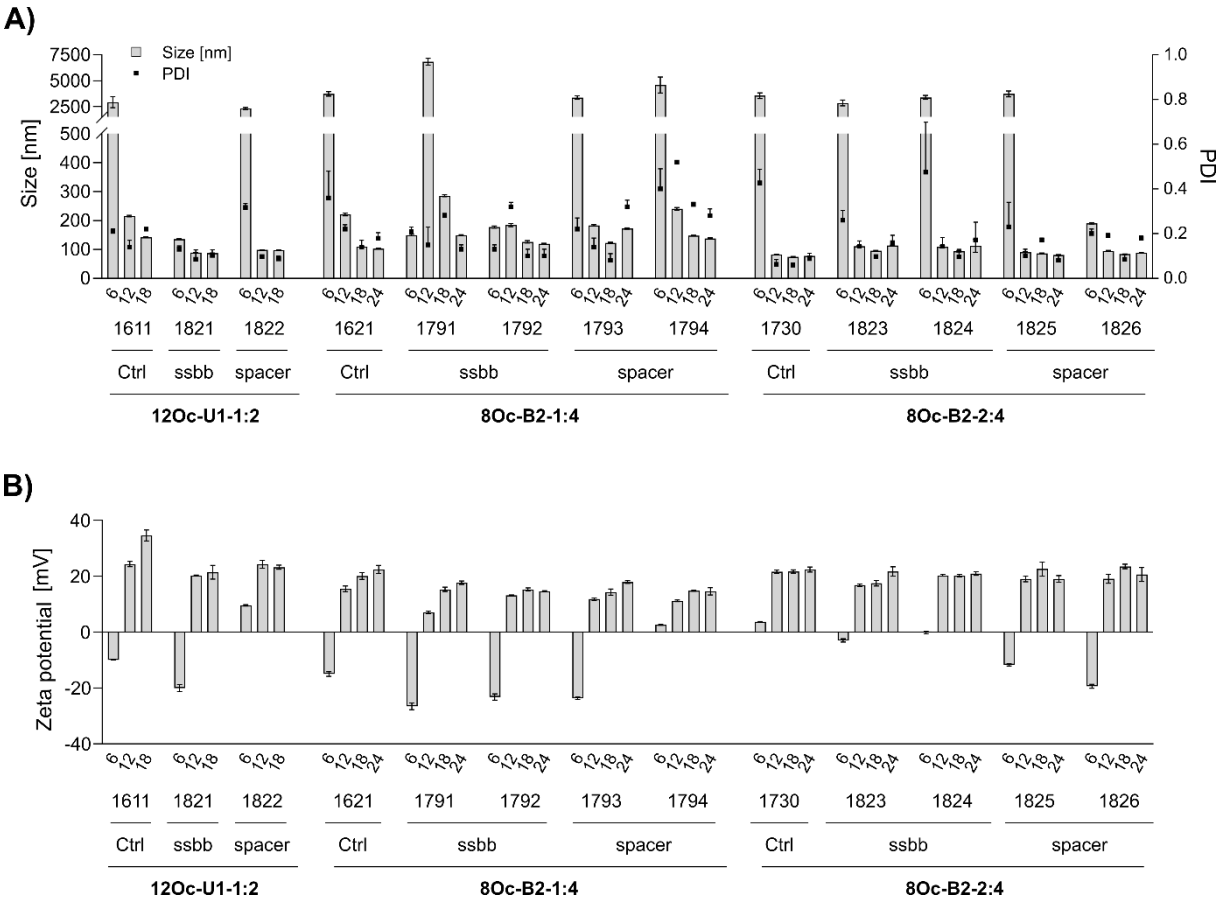



**Figure S2.** DLS and ELS measurements of pDNA polyplexes. Polyplexes were formed with different LAF-Stp carriers at the indicated N/P ratios and a pCMVLuc concentration of 10 µg $mL^{-1}$ (n=3, mean ± SD) and evaluated by A) DLS- measurements for determination of Z-average and PDI and B) ELS measurements to determine zeta potential.

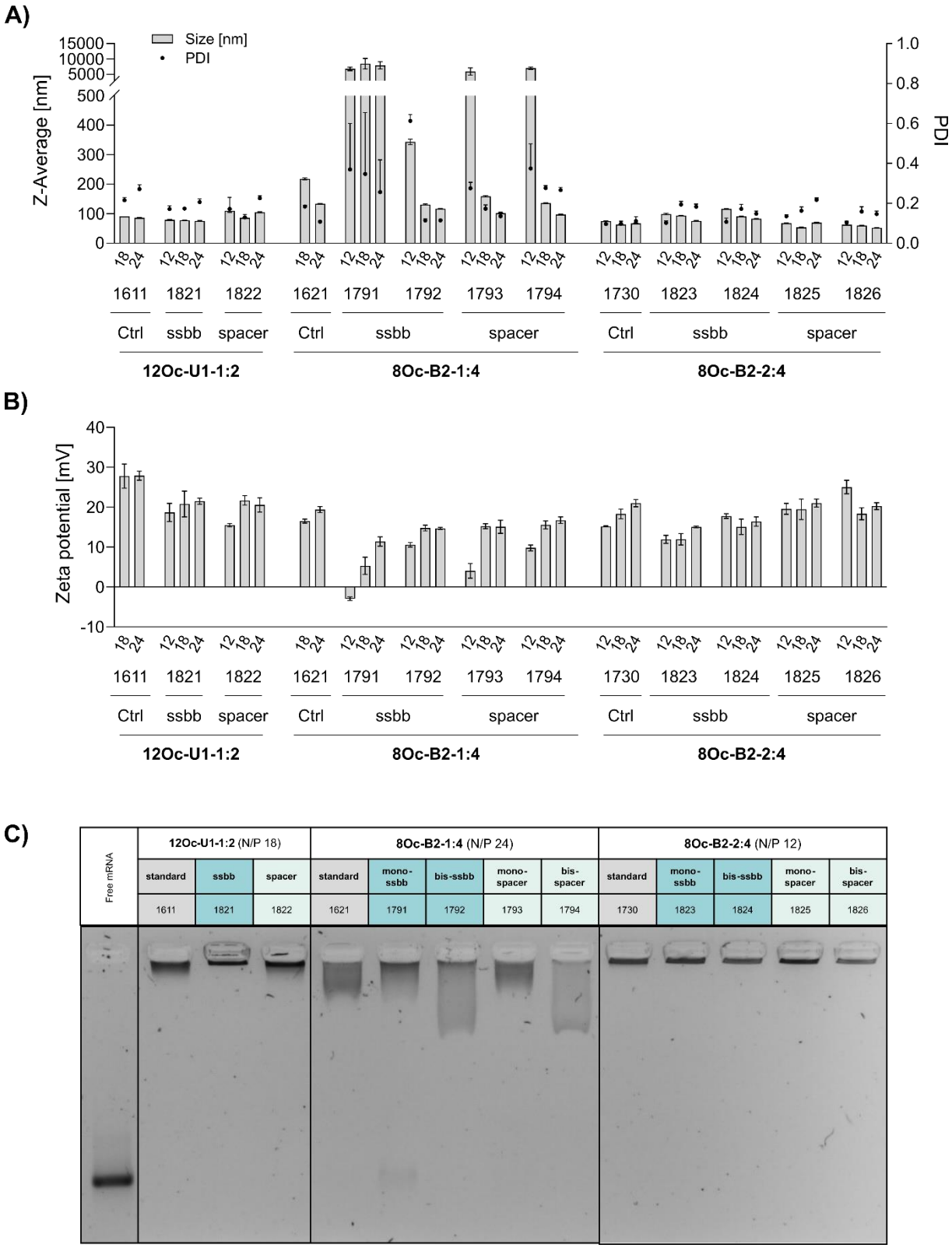



**Figure S3.** Physico-chemical characterization of mRNA polyplexes. Polyplexes were formed with different LAF-Stp carriers at the indicated N/P ratios and an mRNA concentration of 12.5 µg $mL^{-1}$ (n=3, mean ± SD) and evaluated by A) DLS measurements for determination of Z-average and PDI and B) ELS measurements to determine zeta potential. C) Agarose gel shift assay of mRNA polyplexes.

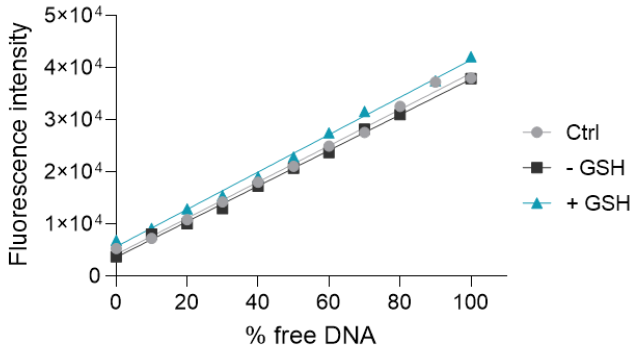



**Figure S4.** Calibration curve for EtBr assay. Free DNA was incubated either for 40 min at RT in HBG (Ctrl) or for 90 min at 37°C with or without GSH (+/- GSH), before addition of ethidium bromide and subsequent measurement.

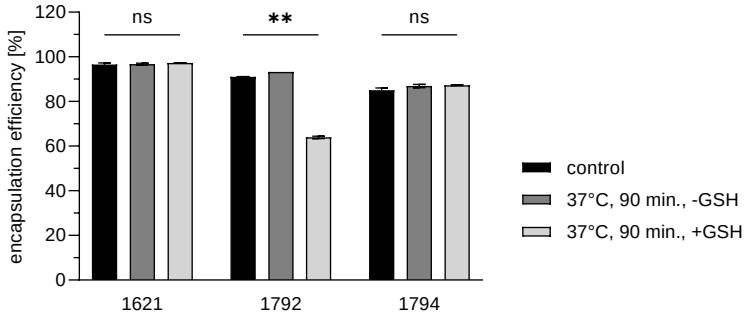



**Figure S5.** Encapsulation efficiency of mRNA polyplexes formed with different LAF-Stp carriers at N/P 24 and a Luc-mRNA concentration of 12.5 µg $mL^{-1}$ (*n*=2). Besides the control group (40 min at RT in absence of GSH), stressed polyplexes (90 min at 37°C +/- GSH) were evaluated. Fluorescence of free mRNA interacting with RiboGreen reagent in intact polyplexes in comparison to polyplexes treated with 1% Triton X-100 and 250 $I.U.mL^{-1}$ heparin.

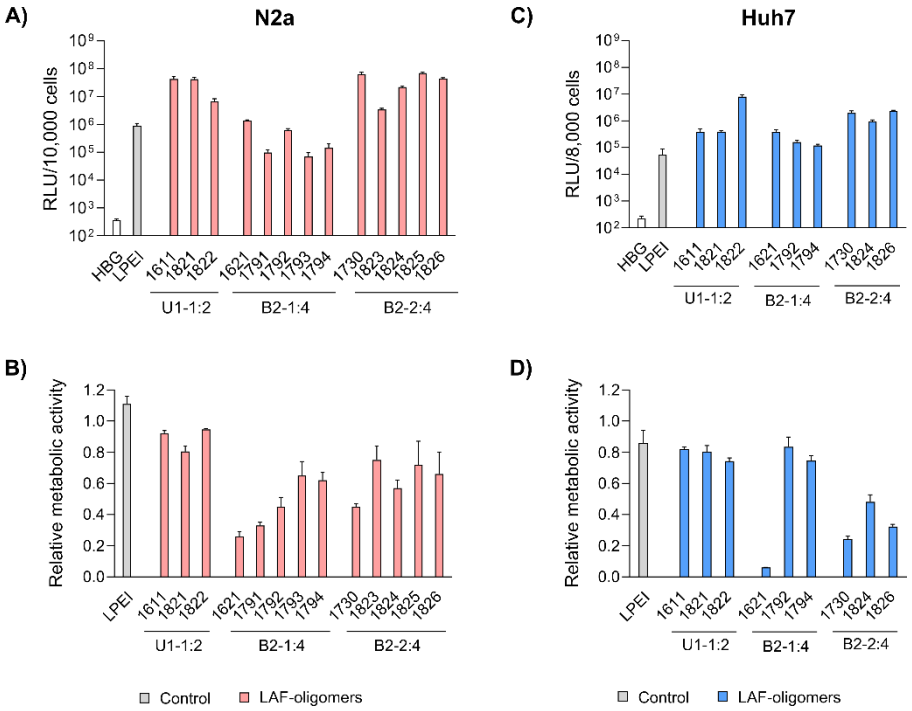



**Figure S6**. *In vitro* screening of pDNA polyplexes containing LAF-Stp oligomers. Polyplexes were formed with the carriers of the library formed at N/P 18 on N2a cells (A and B; 10,000 cells/well) and Huh7 cells (C and D; 8000 cells/well). As control, LPEI (at N/P 6) was transfected. All polyplexes contained 200 ng pDNA per well. Transfection efficiency was determined by luciferase expression assay and cell viability was determined by CellTiter Glo® assay (n=3 +SD), both after 24 hours of incubation.

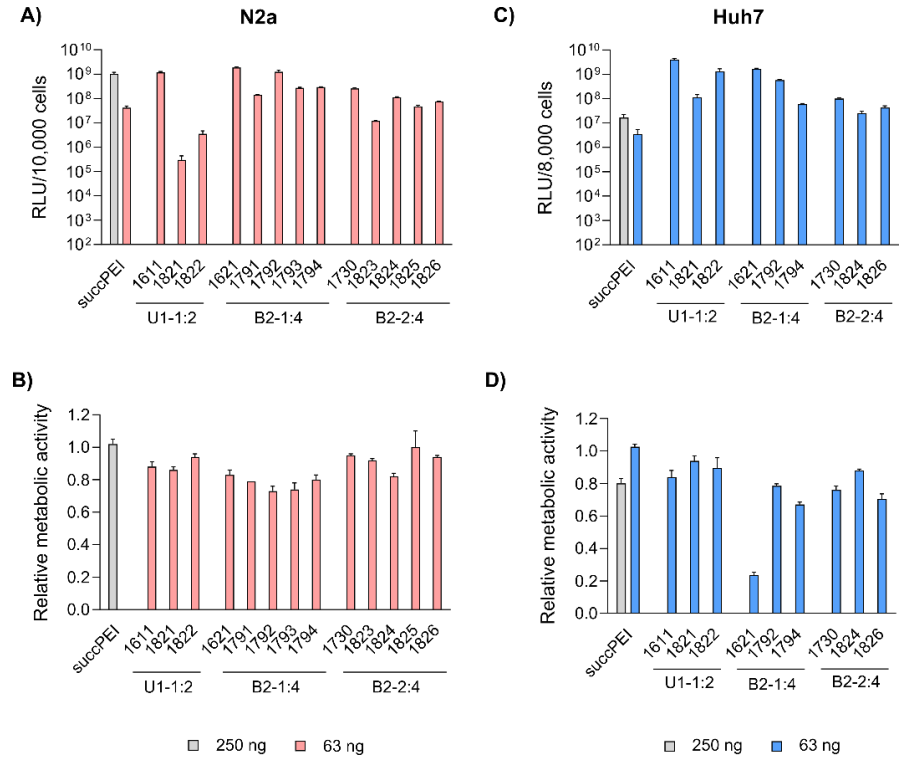



**Figure S7**. *In vitro* screening of mRNA polyplexes containing LAF-Stp oligomers of the library. The polyplexes were formed at N/P 18 (12Oc-U1-1:2 carriers), N/P 24 (8Oc-B2-1:4 carriers) and N/P 12 (8Oc-B2-2:4 carriers) containing 63 ng mRNA per well or with succPEI (w/w ratio 4) containing either 250 ng or 63 ng mRNA per well and transfected to N2a and (A and B; 10,000 cells/well) and Huh7 cells (C and D; 8000 cells/well). Transfection efficiency was determined by luciferase expression assay and cell viability was measured by CellTiter Glo® assay (n=3 +SD), both after 24 hours of incubation.

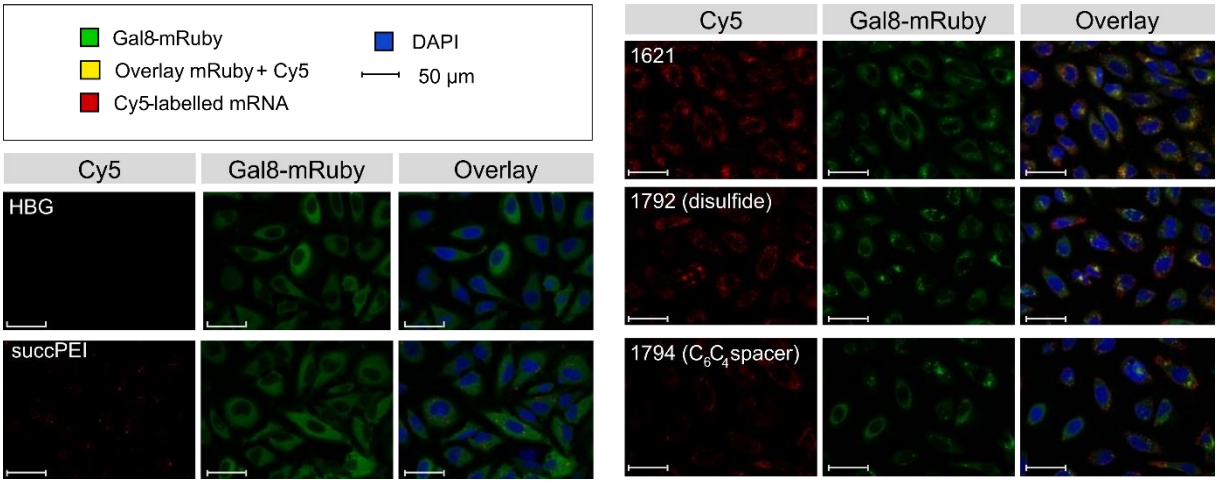



**Figure S8.** Endosomal disruption evaluated in HeLa-Gal8-mRuby cells treated with mRNA-polyplexes formed with the indicated LAF-oligomers in comparison to succPEI (w/w 4) at an mRNA dose of 47 ng at 2 hours after transfection by confocal laser scanning microscopy (CLSM). Punctuate redistribution of cytosolic Gal8–mRuby fluorescence (green) represents endosomal membrane disruption.

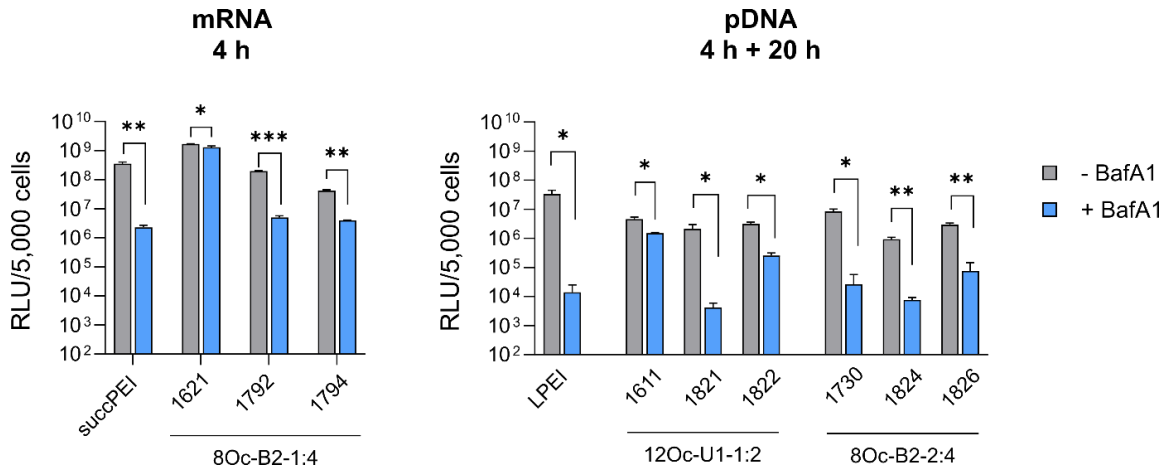



**Figure S9.** Luciferase expression of 5000 HeLa cells/well treated with the indicated polyplexes in absence or presence of Bafilomycin A1 (BafA1) for evaluation of the influence of endosomal acidification on transfection efficiency. The cells were transfected with mRNA or pDNA polyplexes in the presence of 200 nM BafA1. A) The mRNA polyplexes were formed with LAF-carriers of the 8Oc-B2-1:4 topology (1621 – original, 1792 – disulfide, 1794 – spacer) at N/P 24 containing 31 ng Luc-mRNA per well, compared to succPEI (w/w 4, 250 ng mRNA) and incubated for 4 hours in presence of BafA1. Read-out after 4 h via luciferase expression assay (n = 3; mean ± SD). B) pDNA polyplexes were formed with the carriers of either 12Oc-U1-1:2 (1611 – original, 1821 – disulfide, 1822 – spacer) or 8Oc-B2-2:4 (1730 – original, 1824 – disulfide, 1826 – spacer) topology at N/P 18 containing 100 ng pDNA, compared to LPEI (N/P 6, 200 ng). The polyplexes were incubated on the cells in presence of BafA1 for 4 h, followed by medium change and further 20 h of incubation before readout via luciferase expression assay. Significance levels: ns $p > 0.05$; *$p \leq 0.05$; **$p \leq 0.01$; ***$p \leq 0.001$; ****$p \leq 0.0001$.

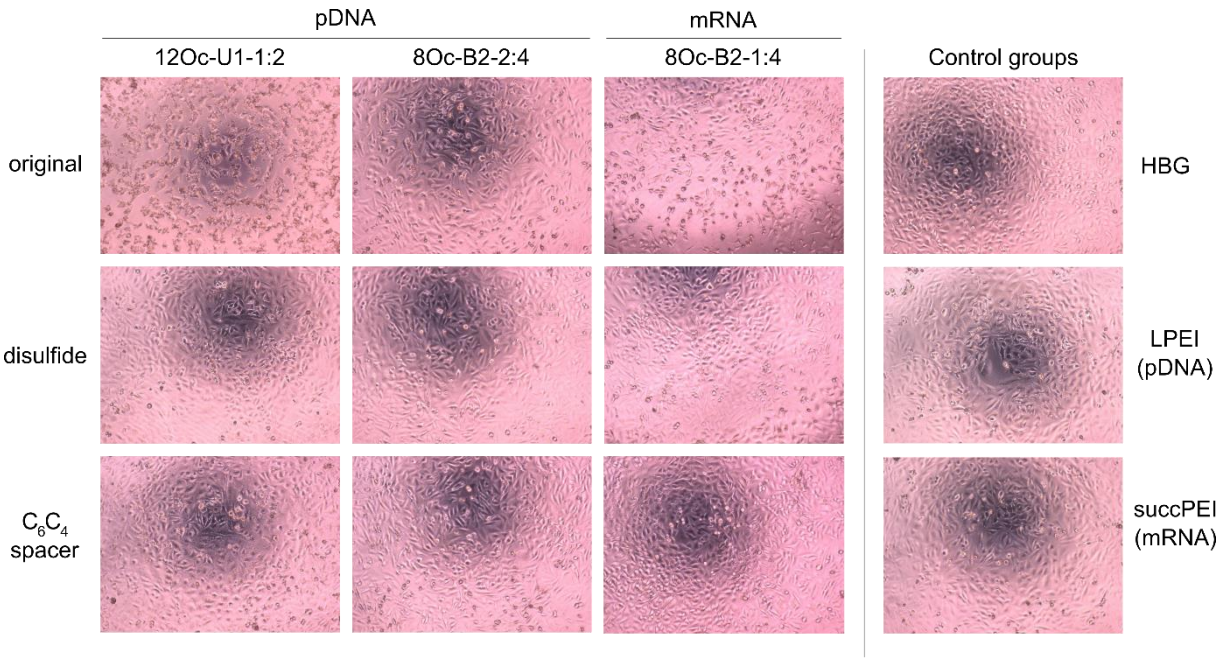



**Figure S10**. Microscopic pictures of HeLa cells at 24 hours after transfection with pDNA- and mRNA-polyplexes in comparison to controls. DNA-polyplexes were formed with the indicated carriers at N/P 18 for LAF-polyplexes and N/P 6 for LPEI (100 ng pDNA/well) and mRNA-polyplexes were formed at N/P 24 for LAF-polyplexes and w/w 4 for succPEI (63 ng mRNA/well). Cells were covered with DMEM low glucose medium.

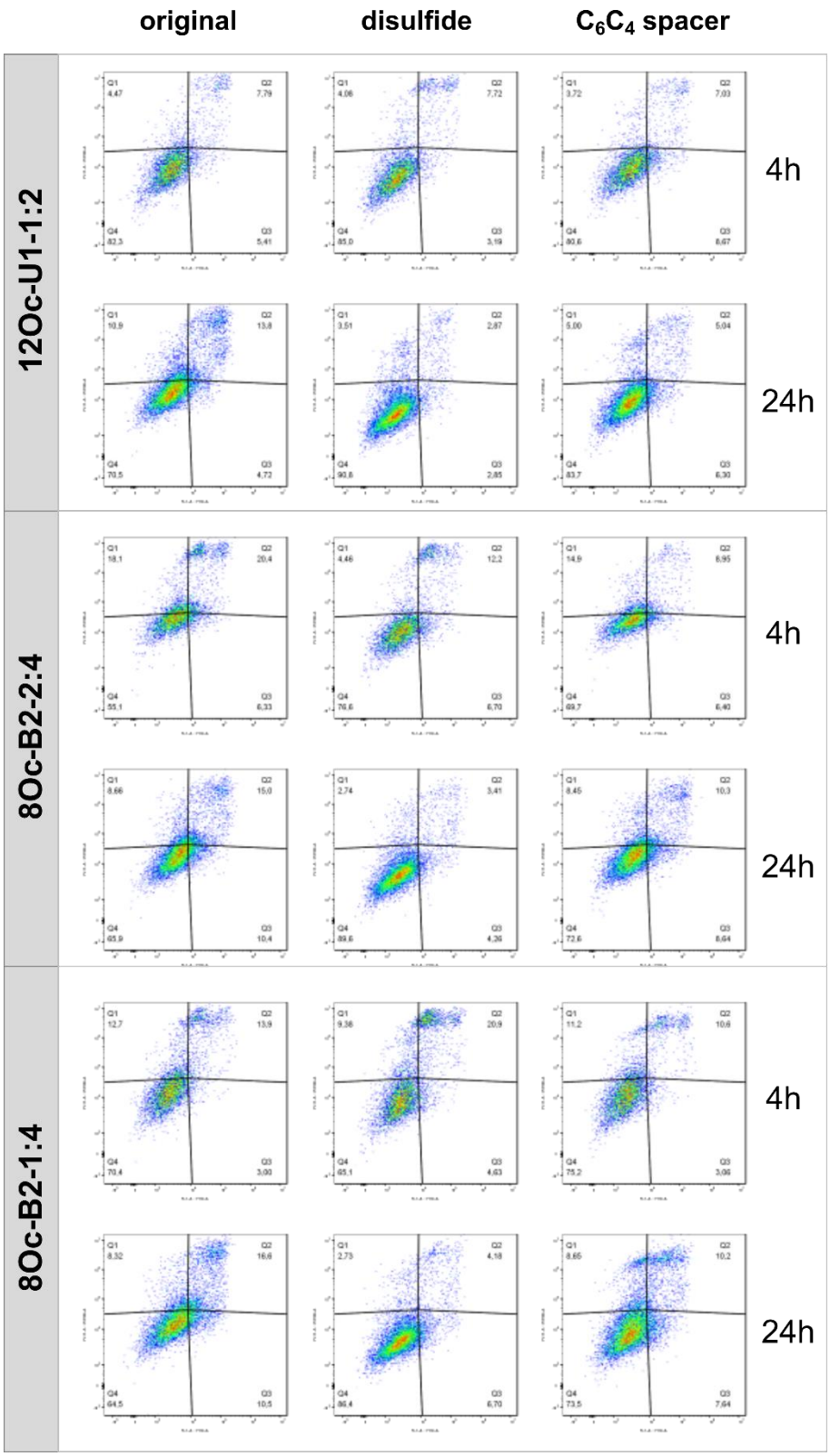



**Figure S11.** Annexin V/propidium iodide assay. Dot plots of flow cytometer analysis of HeLa cells (40,000 cells/well in a 24-well plate) at 4 and 24 hours, respectively after treatment with pDNA polyplexes formed with either 12Oc-U1-1:2-analogs or 8Oc-B2-2:4-analogs at N/P 18 in comparison to HBG-buffer treated cells (125 ng DNA per well) or mRNA polyplexes formed with 8Oc-B2-1:4-analogs at N/P 24 in comparison to HBG-buffer treated cells (78 ng mRNA per well).

## References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3. P. M. Klein, S. Reinhard, D. J. Lee, K. Müller, D. Ponader, L. Hartmann and E. Wagner, *Nanoscale*, 2016, **8**, 18098-18104.
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