Synthesis of a multivalent chelator lipid for stably tethering histidine-tagged proteins onto membranes

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This protocol describes the synthesis of a lipid-like molecule carrying a head group containing two nitrilotriacetic acid moieties. This multivalent chelator lipid can be incorporated into lipid membranes, to which histidine-tagged protein can then be tethered in an oriented fashion. Possible applications of this lipid are protein tethering to solid-supported membranes, to lipid vesicles or to live cells. As compared to conventional monovalent chelator lipids, this lipid can achieve highly stable tethering of proteins by the multivalent chelator head. The eight-step synthesis described in this protocol can be completed within 4–5 weeks.

INTRODUCTION

A large fraction of globular proteins require membrane tethering to fulfill their physiological function. For studying these functions *in vitro*, techniques for tethering proteins to artificial lipid mono- or bilayers are required. To properly mimic the properties of membrane-tethered proteins, oriented and site-specific tethering that avoids large spacers is desired. Chelated metal ions have proven highly versatile for purification and immobilization of histidine-tagged proteins[1]. By incorporation of chelator-functionalized lipid-like molecules into lipid mono- or bilayers, histidine-tagged proteins were successfully tethered in an oriented and reversible fashion[2–9]. A drawback of the traditional chelator lipids based on a single nitrilotriacetic acid (NTA) moiety, however, is its low intrinsic affinity toward the oligohistidine tag, leading to transient and ill-defined attachment[10–12]. Measurement of interactions between membrane-anchored proteins requires stable tethering of proteins by a single histidine tag. Recently, we have reported dramatically improved stability for capturing histidine-tagged proteins by employing multivalent chelator head groups[11,13,14]. Using a chelator head with two NTA moieties (called bis-NTA in this protocol), proteins with a single histidine-tag could be stably tethered onto solid-supported membranes[15,16]. This approach turned out to be highly useful for studying the two-dimensional interaction dynamics on model membranes[15–20].

In this protocol, we summarize the eight-step synthesis of a lipid-like molecule carrying a bis-NTA head group (compound 10, **Fig. 1**). The protected bis-NTA head group (compound 6) is derived from protected amino acids in five steps, followed by coupling to a bis-alkyl amine. Here, the bis-NTA head group is coupled to a secondary amine with two linear C_{18} alkyl groups. In order to ensure high mobility in the membrane and ideal mixing of the chelator lipid with the matrix lipid stearoyl oleoyl phosphocholine (SOPC), we alkylated octadecylamine with octadec-9-enylbromid, yielding an unsaturated bis-alkyl amine (compound 9), which is analogous to a stearoyl-oleoyl lipid. The protocol can also be readily varied in the choice of alkyl groups for obtaining different desired properties of the chelator lipid.

MATERIALS

REAGENTS

- N^{ε}-benzyloxycarbonyl-L-lysine tert-butyl ester (Bachem)
- N^{α}-(benzyloxycarbonyl)-L-glutamic acid (Bachem)
- Octadecylamine (analytical grade)
- Octadec-9-enylbromid (analytical grade)
- Pd/C (10%)
- *tert*-Butyl bromoacetate (analytical grade)
- Triethylamine (TEA) (analytical grade)
- Ethyldiisopropylamin (EDIP, analytical grade)
- O-(benzotriazol-l-yl-N,N,N′,N′-tetramethyluronium tetrafluoroborate (TBTU; analytical grade))
- Ethanedithiol (analytical grade)
- Triisopropylsilane (analytical grade)
- Trifluoroacetic acid (analytical grade)
- Succinic anhydride (analytical grade)
- Organic solvents for reactions: dry chloroform stored on molecular sieves (Fluka), dry dimethyl formamide (DMF) stored on molecular sieves (Fluka), methanol (analytical grade), dry dichloromethane stored on molecular sieves (Fluka)
- Organic solvents for chromatography (analytical grade): chloroform, methanol, ethylacetate, cyclohexane
- Hydrogen gas
- Nitrogen gas

EQUIPMENT

- Synthetic chemistry facilities including hood, rotary evaporator, oil pump; analytical facilities (NMR, mass spectrometry)
- Silica plates for analytical thin layer chromatography (TLC)

PROCEDURE ● TIMING 4–5 weeks

Z-Lys-NTA(OtBu) (compound 2)

1| Dissolve 1.00 g N^{ε}-benzyloxycarbonyl-L-lysine *tert*-butyl ester (compound 1, 2.7 mmol) in dry DMF (25 ml) in a 250-ml round-bottom flask.

2| Add 1.59 ml of *tert*-Butyl bromoacetate (10.8 mmol) and 2.30 ml of EDIPA (13.5 mmol) sequentially. Purge the reaction vessel with nitrogen through a two-way ground joint vacuum take-off on the reaction vessel. Turn on the gas stream while stirring the reaction mixture vigorously. Close both of the in-lets, remove the gas line and stir overnight at 55 °C.

3| Remove the volatiles *in vacuo* at 60 °C and resuspend the partially solidified reaction mixture in 15 ml of cyclohexane:ethylacetate (3:1).

4| Filter the slurry over a sintered glass funnel and wash the precipitate three times with the same solvent (3 × 10 ml).

5| Concentrate the filtrate under reduced pressure and purify by silica gel chromatography with cyclohexane/ethylacetate (3:1) as the mobile phase. The expected yield is 1.3 g (2.3 mmol, 85%) and 1 g is recommended to continue to Step 6.

■ **PAUSE POINT** The product can be stored at −20 °C for at least 6 months.

Lys-NTA(OtBu) (compound 3)

6| Dissolve 1.00 g of compound 2 (1.8 mmol) in 50 ml methanol, purge the solution with nitrogen as described in Step 2 and add 20 mg of 10% Pd/C.

7| Mix the reaction mixture vigorously and stir for 6 h under H_2 atmosphere at room temperature (22–25 °C).

8| Remove the Pd/C catalyst by filtration over celite and then remove the volatiles from the filtrate under reduced pressure. The expected yield is 0.74 g (1.7 mmol, 94%) and 1 g is recommended to continue to Step 9.

■ **PAUSE POINT** The product can be stored at −20 °C for 6 months or more.

Z-bis-NTA(OtBu) (compound 5)

9| Dissolve 1.0 g of compound 3 (2.3 mmol) in 40 ml dry dichloromethane (stored over molecular sieve) and add 0.29 g N^{α}-(benzyloxycarbonyl)-L-glutamic acid (compound 4, 1.0 mmol), 0.96 g TBTU (2.9 mmol) and 0.6 ml EDIPA (3.5 mmol).

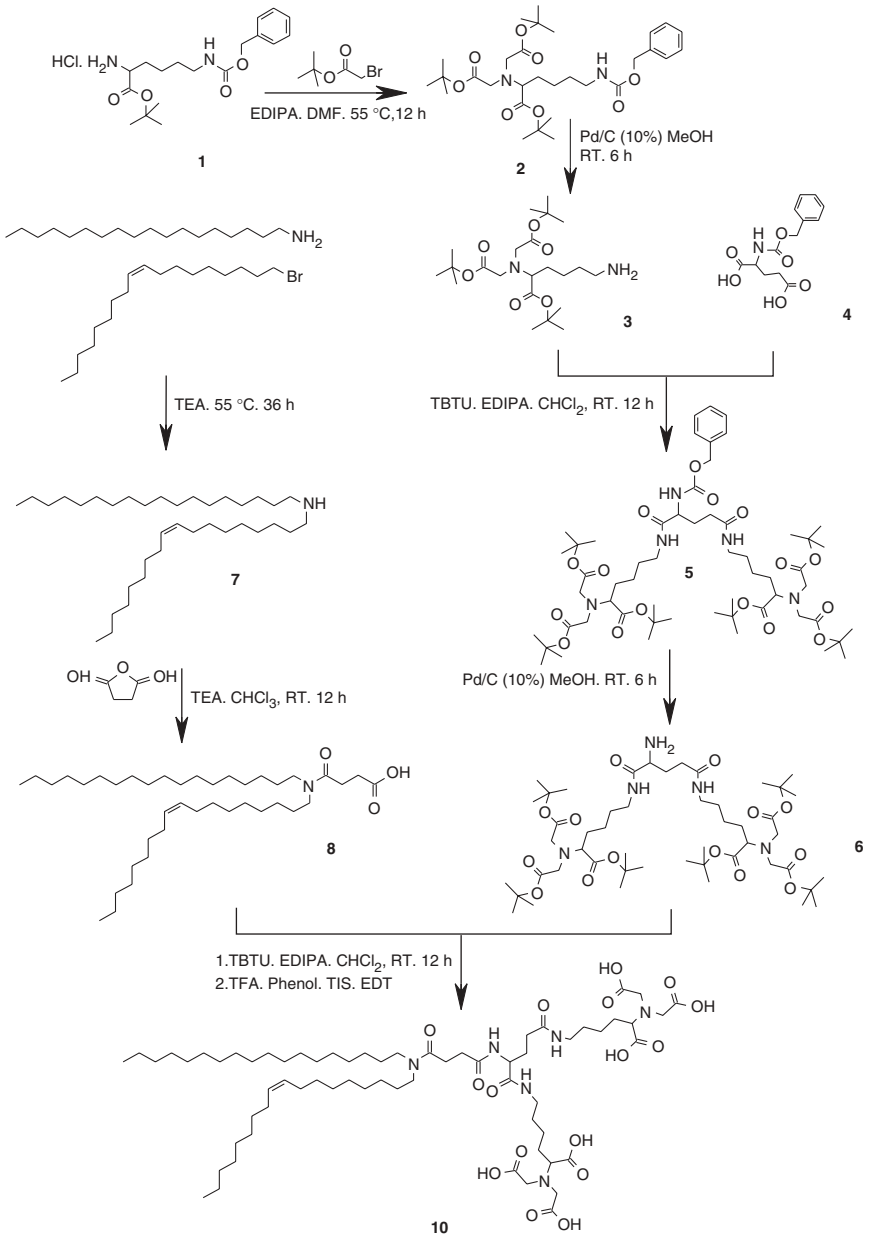

Figure 1 | Synthesis of bis-NTA lipid.

10| Purge the slurry with nitrogen and stir overnight at room temperature. Remove the volatiles under reduced pressure and partition the remaining semi-solid between dichloromethane (100 ml) and MQ water (3 × 30 ml).

11| Dry the organic phase over anhydrous sodium sulphate and remove the volatiles under reduced pressure.

12| Purify the resulting oily mass by silica gel chromatography with ethylacetate/cyclohexane (3:1) as the mobile phase. The expected yield is 0.99 g (0.9 mmol, 90%) and 1 g is recommended to continue to Step 13.

■ **PAUSE POINT** The product can be stored at −20 °C for at least 6 months.

bis-NTA(OtBu) (compound 6)

13| Dissolve 1.00 g of compound 5 (0.90 mmol) in 50 ml methanol and purge the solution with nitrogen.

14| Add 20 mg 10% Pd/C and let the reaction mixture stir for 6 h under hydrogen atmosphere at room temperature.

15| Remove the catalyst by filtration over celite and remove the volatiles under reduced pressure. The expected yield is 0.83 g (0.85 mmol, 94%) and 0.83 g is recommended to continue to Step 25.
■ **PAUSE POINT** The product can be stored at –20 °C for at least 6 months.

Octadec-9-enyl-octadecyl-amine (OEOA) (compound 7)
16| Mix 2.7 g octadecylamine (10 mmol) and 3.3 g 1-bromo-cis-9-octadecen (10 mmol) in the presence of 7 ml TEA (50 mmol) in a round-bottom flask.

17| Purge the reaction vessel with nitrogen and let it stir at 55 °C for 2 d. Dissolve the crude product in 30 ml chloroform and wash 3× with 50 ml water.

18| Concentrate the organic phase and load it on a silica gel column.

19| Elute first the contaminations with 200 ml chloroform, and then the product with 60% chloroform/methanol over five column volumes. The expected yield is 2.1 g (4.05 mmol, 40%) and 1.04 g is recommended to continue to Step 20.
■ **PAUSE POINT** The product can be stored at –20 °C for at least 6 months.

OEOA-Succ (compound 8)
20| Add 0.60 g succinic anhydride (6 mmol) and 2.5 ml TEA (18 mmol) to a solution of 1.04 g of compound 7 (2 mmol) in 50 ml chloroform.

21| Purge the reaction vessel with nitrogen and let it stir overnight at room temperature.

22| Remove the volatiles *in vacuo* and partition the semi solid between chloroform (100 ml) and water (3 × 30 ml).

23| Dry the organic phase by passing over a phase-separatory filter paper.

24| Concentrate the organic phase and precipitate the product with acetone. The expected yield is 1.18 g (1.9 mmol, 95%) and 530 mg is recommended to continue to Step 25.
■ **PAUSE POINT** The product can be stored at –20 °C for at least 6 months.

***Bis*-NTA(OtBu)-OEOA (compound 9)**
25| Dissolve 530 mg of compound 8 (0.85 mmol) in 30 ml dry dichloromethane, and add 834 mg of compound 6 (0.86 mmol).

26| Then add 413 mg TBTU (1.29 mmol) and 0.3 ml EDIPA (1.76 mmol) in this sequence to the solution.

27| Purge with nitrogen gas and stir for 12 h at room temperature.

28| Remove the volatiles under reduced pressure and dissolve the crude product in 100 ml dichloromethane.

29| Using a separatory funnel, wash three times with 30 ml water, and dry the organic phase over anhydrous sodium sulphate.

30| Concentrate it by removing the volatiles under reduced pressure and purify the oily mass by silica gel chromatography with chloroform/ethylacetate (3:2) as eluent. The expected yield is 0.97 g (0.61 mmol, 71 %) and 200 mg is recommended to continue to Step 31.
■ **PAUSE POINT** The product can be stored at –20 °C for at least 6 months.

***Bis*-NTA-OEOA (compound 10)**
31| Dissolve 100 mg phenol, 100 µl triisopropylsilane, 100 µl ethanedithiol and 100 µl water in 25 ml trifluoroacetic acid.
▲ **CRITICAL STEP** Always prepare this solution freshly.

32| Dissolve 200 mg of compound 9 in this solution and stir the reaction mixture at room temperature for 4 h.

33| Remove the volatiles carefully under reduced pressure (oil pump) and dissolve the oily mass again in 5 ml trifluoroacetic acid.

34| Precipitate the product by adding ice-cooled diethylether, remove the supernatant and wash the precipitate with cold diethylether (10 × 15 ml). The expected yield is 370 mg (0.3 mmol, 94%). The product should be stored at −20 °C.

ANTICIPATED RESULTS
All amino acid derivatives with OtBu-protected carboxyl groups (compounds 2, 3, 5, 6 and 9) are obtained as colorless or slightly yellow oily products. After deprotection, the bis-NTA lipid (compound 10) is obtained as a white, crystalline solid with an overall yield of 40–45%. NMR and mass spectrometry (Electron spray ionization (ESI) and/or laser assisted laser ionization

desorption-time of flight (MALDI-TOF) are suitable for analytical characterization of the compounds. In NMR, the signals for the amide protons are particularly helpful for identifying successful reactions by comparison of the integrated signal with the integrated signals for the protons of the OtBu methyl groups. The following NMR signals and m/z (mass to charge ratio) data were obtained:

Z-Lys-NTA(OtBu) (compound 2)
TLC: $R_f = 0.5$ in cyclohexane/ethylacetate (3:1).
^{1}H NMR (250 MHz, $CDCl_3$); δ:
1.44 (s, 18H, $((CH_3)_3COCOCH_2)_2N$-);
1.47 (s, 9H, $(CH_3)_3COCOCH$-);
1.49 (m, 4H, Z-$NHCH_2$-CH_2-CH_2-);
1.59 (m, 2H, Z-NH-$(CH_2)_3CH_2$-);
3.20 (m, 2H, Z-$NHCH_2$-);
3.31 (t, 1H, $((CH_3)_3COCOCH_2)_2NCH$-);
3.46 (dd, 4H, $((CH_3)_3COCOCH_2)_2N$-);
5.07 (s, 2H, $(C_6H_5)CH_2OCONH$-);
5.13 (t, 1H, $(C_6H_5)CH_2OCONH$-);
7.33 (m, 5H, $(C_6H_5)CH_2$-)
MS (MALDI, ESI, $C_{30}H_{48}N_2O_8$): 565 $[MH]^+$.

Lys-NTA(OtBu) (compound 3)
TLC: $R_f = 0.3$ in chloroform/methanol (3:1).
^{1}H NMR (250 MHz, $CDCl_3$); δ:
1.44 (s, 18H, $((CH_3)_3COCOCH_2)_2N$-);
1.47 (s, 9H, $(CH_3)_3COCOCH$-);
1.51 (m, 4H, NH_2CH_2-CH_2-CH_2-);
1.62 (m, 2H, Z-NH-$(CH_2)_3CH_2$-);
2.69 (t, 2H, Z-$NHCH_2$-);
3.31 (t, 1H, $((CH_3)_3COCOCH_2)_2NCH$-);
3.47 (dd, 4H, $((CH_3)_3COCOCH_2)_2N$-);
MS (MALDI, ESI, $C_{22}H_{42}N_2O_6$): 431 $[MH]^+$.

Z-bis-NTA(OtBu) (compound 5)
TLC: $R_f = 0.3$ in cyclohexane/ethylacetate (3:1).
^{1}H NMR (250 MHz, $CDCl_3$); δ:
1.43 (s, 36H, $((CH_3)_3COCOCH_2)_2N$-);
1.45 (s, 18H, $(CH_3)_3COCOCH$-);
1.47–1.65 (m, 12H, $(CH_3)_3COCOCH(CH_2)_3$-);
1.96–2.04 (m, 2H, Z-$NHCHCH_2CH_2$-);
2.22–2.30 (m, 2H, Z-$NHCHCH_2$-);
3.14–3.22 (m, 6H, and $((CH_3)_3COCOCH_2)_2NCH(CH_2)_3CH_2$-);
3.46 (dd, 8H, $((CH_3)_3COCOCH_2)_2N$-);
4.17 (m, 1H, Z-NHCH-);
5.03 (s, 2H, $(C_6H_5)CH_2OCONH$-);
6.40 (d, 1H, Z-NH); 6.53 (t, 1H Z-NHCHCONH-);
6.99 (t, 1H Z-$NHCH(CH_2)_2CONH$-);
7.28 (m, 5H, $(C_6H_5)CH_2$-);
MS (MALDI, ESI, $C_{57}H_{95}N_5O_{16}$): 1107 $[MH]^+$.

bis-NTA(OtBu) (compound 6)
TLC: $R_f = 0.3$ in chloroform/methanol (5:2).
^{1}H NMR (250 MHz, $CDCl_3$); δ:
1.43 (s, 36H, $((CH_3)_3COCOCH_2)_2N$-);
1.45 (s, 18H, $(CH_3)_3COCOCH$-); 1.47-1.65 (m, 12H, $(CH_3)_3COCOCH(CH_2)_3$-);
2.0–2.1 (m, 2H, Z-$NHCHCH_2$-);
2.37–2.44 (m, 2H, Z-$NHCHCH_2CH_2$-);
3.2–3.3 (m, 6H, and $((CH_3)_3COCOCH_2)_2NCH(CH_2)_3CH_2$-);
3.36–3.50 (dd, 8H, $((CH_3)_3COCOCH_2)_2N$-);

3.56 (t, 1H, Z-NHC**H**-);
5.03 (s, 2H, $(C_6H_5)C\mathbf{H}_2OCONH$-);
6.88 (t, 1H Z-NHCH$(CH_2)_2$CON**H**-);
7.68 (t, 1H Z-NHCHCON**H**-);
MS (MALDI, ESI, $C_{49}H_{89}N_5O_{14}$): 973 $[MH]^+$.

Octadec-9-enyl-octadecyl-amine (OEOA) (compound 7)
^{1}H NMR (250 MHz, $CDCl_3$); δ:
0.89 (t, 6H, $C\mathbf{H}_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5CH_2CHCHCH_2(CH_2)_6C\mathbf{H}_3$);
1.27 (s, 52H, $CH_3(C\mathbf{H}_2)_{15}CH_2CH_2NHCH_2CH_2(C\mathbf{H}_2)_5CH_2CHCHCH_2(C\mathbf{H}_2)_6CH_3$);
1.91 (q, 4, $CH_3(CH_2)_{15}C\mathbf{H}_2CH_2NHCH_2C\mathbf{H}_2(CH_2)_5CH_2CHCHCH_2(CH_2)_6CH_3$);
2.01 (q, 4, $CH_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5C\mathbf{H}_2CHCHC\mathbf{H}_2(CH_2)_6CH_3$);
2.94 (t, 4, $CH_3(CH_2)_{15}CH_2C\mathbf{H}_2NHC\mathbf{H}_2CH_2(CH_2)_5CH_2CHCHCH_2(CH_2)_6CH_3$);
5.36 (q, 2, $CH_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5CH_2C\mathbf{H}C\mathbf{H}CH_2(CH_2)_6CH_3$).
MS (MALDI, ESI, $C_{36}H_{73}N$); MH^+ 521.

OEOA-Succ (compound 8)
^{1}H NMR (250MHz, CDCl3); δ:
0.88 (t, 6H, $C\mathbf{H}_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5CH_2CHCHCH_2(CH_2)_6C\mathbf{H}_3$);
1.27 (s, 52H, $CH_3(C\mathbf{H}_2)_{15}CH_2CH_2NHCH_2CH_2(C\mathbf{H}_2)_5CH_2CHCHCH_2(C\mathbf{H}_2)_6CH_3$);
1.54 (q, 4H, $CH_3(CH_2)_{15}C\mathbf{H}_2CH_2NHCH_2C\mathbf{H}_2(CH_2)_5CH_2CHCHCH_2(CH_2)_6CH_3$);
2.00 (q, 4H, $CH_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5C\mathbf{H}_2CHCHC\mathbf{H}_2(CH_2)_6CH_3$);
2.69 (t, 4H, $CH_3(CH_2)_{15}CH_2C\mathbf{H}_2NHC\mathbf{H}_2CH_2(CH_2)_5CH_2CHCHCH_2(CH_2)_6CH_3$);
3.22 (t, 2H, $OHCOCH_2C\mathbf{H}_2CONH$-);
3.30 (t, 2H, $OHCOC\mathbf{H}_2CH_2CONH$-);
5.34 (q, 2, $CH_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5CH_2C\mathbf{H}C\mathbf{H}CH_2(CH_2)_6CH_3$).
MS (MALDI, ESI, $C_{40}H_{77}NO_3$); MH^+ 620.

***Bis*-NTA(OtBu)-OEOA (compound 9)**
TLC: $R_f = 0.6$ in chloroform/ethylacetate (3:2)
^{1}H NMR (250MHz, CDCl3); δ:
0.87 (t, 6H, $C\mathbf{H}_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5CH_2CHCHCH_2(CH_2)_6C\mathbf{H}_3$);
1.25 (s, 52H, $CH_3(C\mathbf{H}_2)_{15}CH_2CH_2NHCH_2CH_2(C\mathbf{H}_2)_5CH_2CHCHCH_2(C\mathbf{H}_2)_6CH_3$);
1.45 (s, 36H, $((C\mathbf{H}_3)_3COCOCH_2)_2N$-); 1.46(s, 18H, $(C\mathbf{H}_3)_3COCOCH$-);1.47-3.5 (H);
4.37 (m, 1H, NHC**H**-);
5.34 (q, 2, $CH_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5CH_2C\mathbf{H}C\mathbf{H}CH_2(CH_2)_6CH_3$);
6.58 (t, 1H, N**H**); 7.42 (t, 1H, N**H**); 7.61 (t, 1H, N**H**).
MS (MALDI, ESI, $C_{89}H_{164}N_6O_{16}$); MNa^+ 1592.

Bis-NTA-OEOA (compound 10)
^{1}H NMR (250MHz, CDCl3); δ:
0.87 (t, 6H, $C\mathbf{H}_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5CH_2CHCHCH_2(CH_2)_6C\mathbf{H}_3$);
1.25 (s, 52H, $CH_3(C\mathbf{H}_2)_{15}CH_2CH_2NHCH_2CH_2(C\mathbf{H}_2)_5CH_2CHCHCH_2(C\mathbf{H}_2)_6CH_3$);
1.47–3.5 (H); 5.03 (m, 1H, NHC**H**-);
5.34 (q, 2, $CH_3(CH_2)_{15}CH_2CH_2NHCH_2CH_2(CH_2)_5CH_2C\mathbf{H}C\mathbf{H}CH_2\ (CH_2)_6CH_3$).
MS (MALDI, ESI, $C_{65}H_{111}N_6O_{16}$); MH^+ 1233.

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

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