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Anion transport in liposomes responds to variations in the anchor chains and the fourth amino acid of heptapeptide ion channels

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Seven heptapeptide derivatives have been prepared. The peptide structure is $(Gly)_3Xxx(Gly)_3$ in which Xxx stands for a variable amino acid. The amino acid variations include azetidine carboxylic acid, pipecolic acid, *meta*-aminobenzoic acid, proline, and leucine. All seven compounds have a *C*-terminal benzyl group. In all cases, the heptapeptide's *N*-terminus was linked to diglycolic acid and a dialkylamine. In five cases, the *N*-terminal group was didecylamine and in two cases, *N*-ethyl-*N*-decyl. Chloride and carboxyfluorescein release from phospholipid vesicles was studied with the result that $C_{10}H_{21}N(C_2H_5)COCH_2OCH_2CO–NH–(Gly)_3Leu(Gly)_3–OCH_2Ph$ was the most active. Hill analysis showed that this compound involves pore formation by four monomer units rather than two, as previously found for other members of this family.

Introduction

The recent appearance of protein channel crystal structures has dramatically increased the amount of structural information available about these ion and molecule transporters. The importance of such structures is apparent from the award of the 2003 Nobel Prize in Chemistry for the accomplishment.[1] Important as such structures are, they do not provide direct information about interactions with the membrane or the mechanism(s) of ion transport. Synthetic organic channels[2] are more amenable to structure–activity studies because of their smaller size and the fact that the most successful ones were designed to be modular.[3]

In previous work, we developed a successful cation-transporting channel compound.[4] Although it was designed to be a model for proteins, it is a true channel. Members of this "hydraphile" family insert in the phospholipid bilayer and transport ions effectively and with some selectivity. The synthetic channels lack the highly developed selectivity filters and rectification behavior of protein channels[5] but the synthetic versions transport ions and show open–closed behavior[6] characteristic of proteins. These synthetic models are, in fact, functioning ion channels although they are clearly less evolved than their protein counterparts.

Quite recently, we designed an amphiphilic peptide intended to be a chloride ion transporter. It was constructed from four key modules. The central element is a heptapeptide that has the sequence $(Gly)_3Pro(Gly)_3$. The sequence was based on the conserved Gly-Xxx-Xxx-Pro sequence identified in the CLC family of chloride transporters. The simplest such sequence is Gly-Gly-Gly-Pro (GGGP). Addition of three glycine residues gives a heptapeptide with the sequence GGGPGGG. Two modules anchored the *N*-terminal end of this peptide. The *N*-terminus comprises a dialkylamide, $R_2NCO–$. The initial anchors chosen were octadecyl chains. These were intended to interact with the phospholipid's fatty acyl chains. The hydrocarbon anchors are connected to the heptapeptide by diglycolic acid. This diacid was chosen for convenience and because its polar residues mimic the midpolar regime of phospholipid monomers. Finally, the *C*-terminus was esterified by benzyl alcohol. The latter was used to prevent ionization of the *C*-terminal carboxyl and to permit future manipulations because it can be removed hydrogenolytically.

The amphiphilic peptide[7] $(C_{18}H_{37})_2NCOCH_2OCH_2CONH–(Gly)_3Pro(Gly)_3OCH_2Ph$[8] showed remarkable properties when it inserted into phospholipid bilayers.[9] In a previous study reported in this Journal, we showed that changing the central amino acid in the heptapeptide from proline to pipecolic acid dramatically altered the ion transport profile of this compound.[10] We now report the results of systematic variation of that fourth residue and its effect on transport efficacy.

Results and discussion

Pipecolic acid and proline are homologs. They have the same structural features and stereochemistry and differ only in ring size. Our expectation was that changing the $(Gly)_3$-Pro-$(Gly)_3$ sequence to $(Gly)_3$-Pip-$(Gly)_3$ would lead to a minor variation in the peptide's bend angle and a commensurate small change in anion release activity. Instead, we found that release from phospholipid vesicles of the fluorescent dye carboxyfluorescein was significantly diminished when a single methylene group expanded the proline ring.

Compounds studied

The compounds prepared for the present report are shown as **1–7**. In all cases, the compounds possess four "modules." The *N*-terminus comprises a pair of hydrocarbon chains [either ethyl and decyl (**1** and **4**) or didecyl (**2**, **3** and **5–7**)]. These

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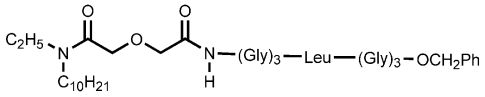
hydrocarbon chains are expected to insert in the phospholipid bilayer and anchor the compound therein. The dialkylamino group is linked to the heptapeptide by a diglycolic acid residue. The *C*-terminus of the heptapeptide is benzyl in **1–7**. The principal variations in structure are changes in the fourth amino acid residue of the heptapeptide chain. They are: leucine (Leu, L) in **1** and **2**, azetedinecarboxylic acid in **3**, proline (Pro, P) in **4** and **5**, pipecolic (Pip) acid in **6**, and 3-aminobenzoic acid in **7**. The stereochemistry in the central amino acid of compounds **1–6** is the same. Although 3-aminobenzoic acid is not one of the common natural amino acids, it has amino and carboxyl groups disposed at 120° to each other. The use of this compound also permitted us to assess the influence, if any, of an aromatic residue within the heptapeptide chain.

1



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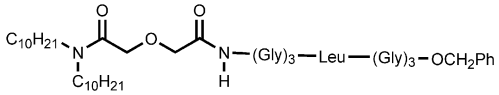

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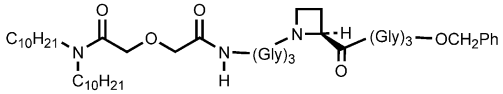

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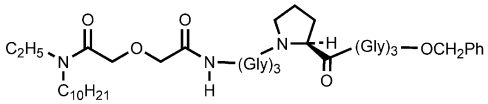

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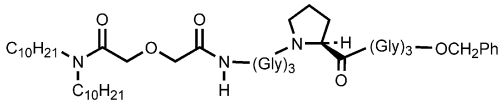

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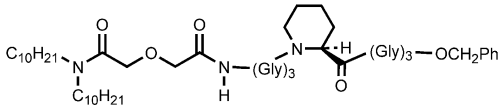

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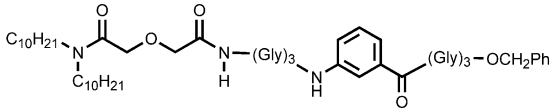
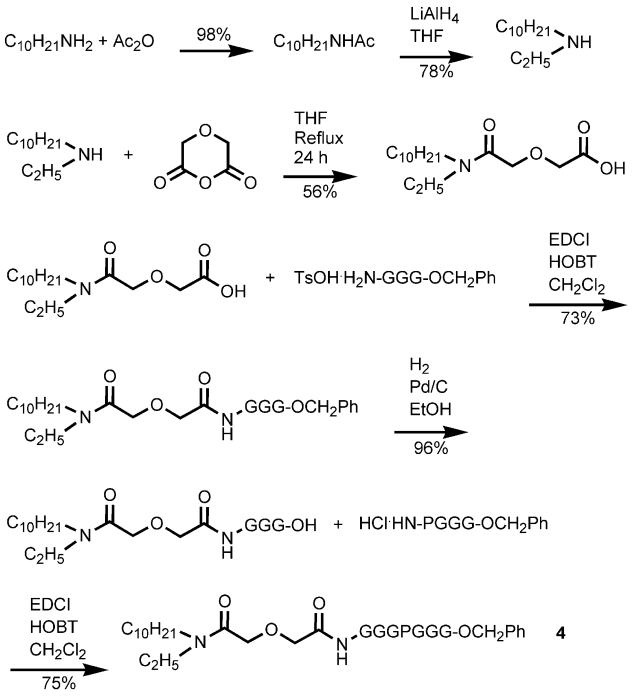


The amphiphilic peptides were prepared in a series of steps beginning at the heptapeptide's *N*-terminus. The dialkylamine and diglycolic anhydride were heated at reflux in toluene or THF for 48 h. The resulting acid, $R_2NCOCH_2OCH_2COOH$, was coupled to triglycine by standard carbodiimide methods to give $R_2NCOCH_2OCH_2CONH$–Gly-Gly-Gly–OR′. The second segment, which contained various amino acids (Aaa) in the incipient fourth position, $H_{(1,2)}N$–Aaa-Gly-Gly-Gly–OCH_2Ph was then coupled to the deprotected fragment. The compounds obtained had spectral properties that corresponded to their assigned structures and the following melting points: **1**, 179–181 °C; **2**, 186–188 °C; **3**, 117–119 °C; **4**, 195 (dec) °C; **5**, 127–128 °C; **6**, 78–80 °C; and **7**, >205 (dec) °C. The synthetic sequence used for the preparation of **4** is shown in Scheme 1.



Scheme 1

Chloride release from phospholipid vesicles

The efficacy of ion transport was assayed by using a chloride selective electrode to detect efflux directly from phospholipid vesicles.[11] The liposomes were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA) (20 mg, 7 : 3 w/w) by the lipid film method.[12] The vesicle suspension was filtered through a 200 nm filter membrane (5 times) using a mini-extruder. The suspension was then passed through a Sephadex G25 column, which had been equilibrated with external buffer (400 mM K_2SO_4 : 10 mM HEPES, pH adjusted to 7.0). Analysis by light scattering showed that the vesicle diameter was consistently ~200 nm. The final lipid concentration was confirmed by colorimetric determination of the phospholipid–ammonium ferrothiocyanate complex.[13]

Chloride release was determined by recording the voltage output of a chloride selective (Accumet Chloride Combination) electrode. The electrode and vesicle suspension were allowed to equilibrate for five minutes, after which aliquots containing the compound of interest (~9 mM in 2-propanol) were added. Ion release was monitored for approximately 30 min in each case; data for 1500 s are presented in the graphs. After 0.5 h, complete lysis of the vesicles was induced by addition of a 2% aqueous solution of Triton X-100 (100 μL) and the data previously recorded were normalized to this maximal value. The measured transport rates are shown in Fig. 1. Each curve shown is the average of 3–7 different experiments done with [lipids] = 0.31 mM and [compound] = 65 μM.

The upper panel of Fig. 1 shows chloride release data for compounds **1**, **4**, and **5**. In compounds **1** and **4**, the dialkylamino groups are $CH_3CH_2N(CH_2)_9CH_3$ and in **5** it is didecylamino. Amphiphiles **1** and **4** are identical except for the fourth amino acid (Leu *vs.* Pro) and **4** and **5** are identical except for the dialkylamino group. A comparison of **1** and **4** reveals that release of chloride ion when **1** is present is much greater than that induced by a comparable amount of **4**. In both cases, however, the initial portions of the curve are steep, suggesting that insertion occurs quite readily. This result contrasts with the more gradual initial rate observed for **5**. The flat portions of the release curves for **1** and **4** are clearly offset but nearly parallel. Although Cl^- release begins more gradually with **5**, the rate of increase is greater at 1500 s than for **4**. Essentially complete Cl^- release is observed for **1** at 1500 s.

We note two technical issues with respect to the chloride ion release study. First, the response of the chloride electrode is

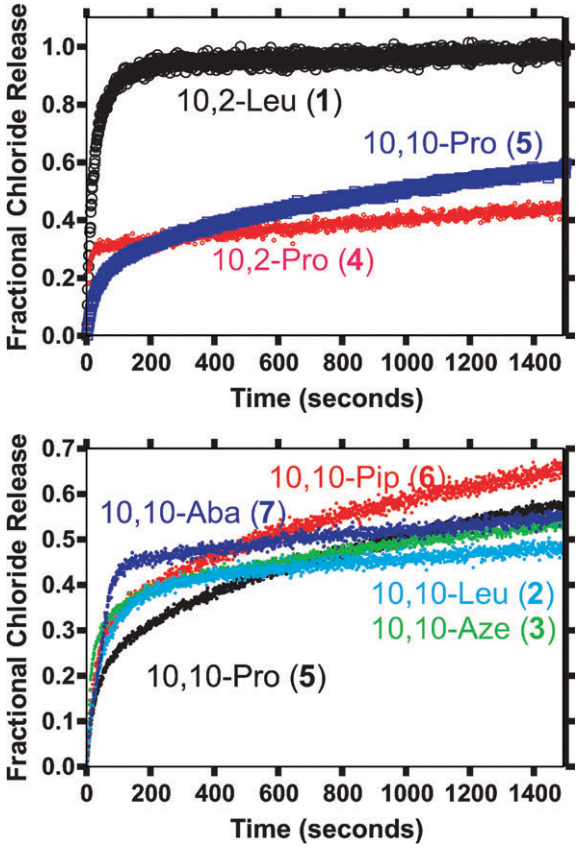


Fig. 1 (Upper panel) Fractional chloride release from phospholipid vesicles by **1**, **4**, and **5**. (Lower panel) Fractional chloride release from phospholipid vesicles by **2–7**. See text for concentration details.

slow. Ion release is most rapid at the initial stage when the system is equilibrating. This presumably most affects the exponential portion of the release curve. Second, the linear portion of the curve has a shallow slope at the lowest ionophore concentrations. Following these profiles to full release would require considerable time and greater liposome stability than the system currently exhibits. We further note that the graphical representations shown in the figures are either averages of three or more independently acquired data sets or representative data sets that clearly portray the average result. In all cases, more data were obtained than are shown in the figures and none contradicts the representations included herein.

Ion release from phospholipid vesicles occurs as soon as a pore is formed in the bilayer.[14] However pore formation occurs, it seems reasonable that the amphiphiles having a single long chain require less pre-organization to insert into the bilayer. A comparison of the initial release profiles comports with this expectation. It also seems reasonable that the ultimate efficacy of the pore would vary when the alkyl chains differ. The difference in Cl^- release observed for **4** and **5** suggests that while insertion and pore formation is apparently more rapid for **4**, the resulting pore is ultimately superior for **5**.

The data presented in the lower panel of Fig. 1 compare the five *N*,*N*-didecyl heptapeptide derivatives. Compound **5** is included in both graphs and permits comparison even though the ordinates differ. As in the upper panel of this figure, we note that there are differences in curve shape. Overall, however, the five compounds all show similar Cl^- release rates. The inclusion of **5** in the upper panel shows that Cl^- release is similar for it and for **4**. The similarity in Cl^- release rates seems reasonable considering the small structural differences that exist in the fourth amino acids. The key exception is compound **1**, which is by far the best Cl^- transporter in this study. It is discussed further below.

Release of the carboxyfluorescein anion from vesicles

In previous studies, we found that chloride release from phospholipid vesicles was mediated by $(C_{18}H_{37})_2NCO$-CH_2OCH_2CO–NH–GGGPGGG–OCH_2Ph (**8**) and by its –GGGLGGG– analog (**9**). Anion transport by the latter was about 6-fold lower at an identical concentration than when the central amino acid was proline.[10] This led us to assess the effect of pipecolic acid in the center of the heptapeptide. In those studies, transport of carboxyfluorescein (CF) through a phospholipid bilayer was also measured. Carboxyfluorescein is an anionic dye that self quenches when it is encapsulated at sufficiently high concentrations within liposomes. If the anion is released from the vesicles, its external concentration is lower and fluorescence is readily and quantitatively detected. The concentration-dependent release of CF by **1** is shown in Fig. 2.

In the earlier study,[10] we found about a 3-fold difference in CF transport by the bis(octadecyl) heptapeptides when the central amino acid was proline (faster) or pipecolic acid (slower). A nearly 30-fold difference in CF transport between 10,10-Pro (**5**) and 10,10-Pip (**6**, slower) was also noted. The results presented here for chloride transport show much less difference between **5** and **6** than was observed when the anion was CF. Indeed, there is relatively little difference in the chloride transport rates for **2–7**, although **1** is dramatically different. The high chloride transport rate exhibited by $C_{10}H_{21}NEtCOCH_2OCH_2CONH$–$(Gly)_3Leu(Gly)_3OCH_2Ph$ (**1**) compared to **2–7** demanded that a concentration dependence study be undertaken. This was done and is shown in Fig. 2. A reasonable concentration dependence of CF transport by **1** was observed for a ~7-fold concentration range from 25.9–189 μM.

By using the concentration dependent fluorescence data for **1** (Fig. 2) we prepared a Hill plot to ascertain if pores formed from **1** involved more than 2 monomers, as found for previous compounds in this series. The data are shown in Fig. 3.

The slope of the Hill plot is 3.8 ± 0.7, suggesting that twice as many monomers are involved in pore formation by **1** than the dimer assembly found for either **5** or previously for **8**. It seems reasonable that if more monomers associate to form a pore, the pore would be larger than if fewer monomers were present. This, in turn, should afford a larger pore, resulting in a higher ion flux. Overall, we expect leucine-containing **1** and **2** to be the most flexible and adaptable. Of these, only **1** combines a single decyl chain with the flexible leucine. We have already shown in previous work that anchor chain length is an important variable in pore-forming ability by these compounds.[15] It appears that **1** has a unique combination of flexibility and insertion dynamics to permit facile pore formation and complete anion release.

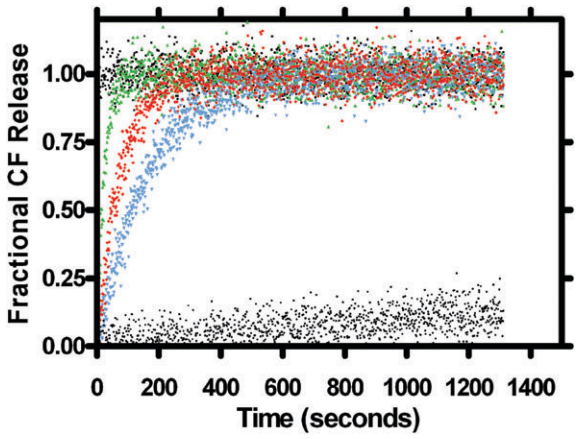


Fig. 2 Concentration dependent, fractional carboxyfluorescein release from phospholipid vesicles (see text for details). Concentrations of **1** are, from bottom to top, 25.9, 64.5, 99.0, 127.0, and 189 μM.

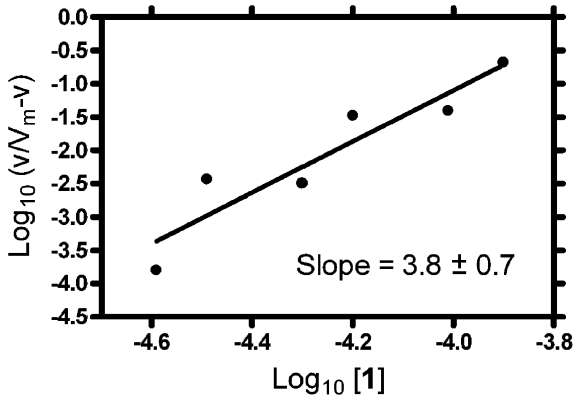


Fig. 3 Hill plot showing a slope indicating an aggregation of four monomers active in pore formation. See text for details.

Conclusion

Seven heptapeptides have been prepared that have either two decyl chains or a decyl and an ethyl chain at their *N*-terminal ends. The fourth (central) amino acid in the heptapeptide sequence has been varied from cyclic to acyclic (leucine). The cyclic amino acids include proline and its 4- and 6-membered ring analogs. In addition, the unnatural amino acid *meta*-aminobenzoic acid has been incorporated into the peptide chain. Chloride release mediated by each of these compounds was measured and found generally to be similar, except for the –GGGLGGG– derivative having ethyl and decyl *N*-terminal groups. When carboxyfluorescein release by unusually active compound **1** was studied, Hill plot analysis showed that four monomers were involved in pore formation. Previous studies with identical, twin-chained analogs showed that only two amphiphiles were involved in pore formation. We speculate that the larger number of monomers leads to a larger pore and a higher anion release rate. Whether the difference in monomer number affects ion selectivity was not addressed and will be part of a future effort to better understand pore formation by these ionophores.

General methods

All reaction solvents were freshly distilled and reactions were conducted under N_2 unless otherwise stated. Et_3N was distilled from KOH and stored over KOH. CH_2Cl_2 was distilled from CaH_2. Column chromatography was performed on silica gel 60 (230–400 mesh). Thin layer chromatography was performed with silica gel 60 F_{254} plates with visualization by UV light (254 nm) and/or by phosphomolybdic acid (PMA) spray. Starting materials were purchased from Aldrich Chemical Co., and used as received. ^{1}H-NMR spectra were recorded at 300 MHz in $CDCl_3$ unless otherwise noted and are reported as follows: Chemical shifts (ppm δ, internal Me_4Si) [integrated intensity, multiplicity (b = broad; s = singlet; d = doublet; t = triplet; m = multiplet, bs = broad singlet, *etc.*), coupling constants in hertz, assignment]. ^{13}C-NMR spectra were obtained at 75 MHz in $CDCl_3$ unless otherwise noted and referenced to $CDCl_3$ (δ 77.0). Infrared spectra were recorded in KBr unless otherwise noted and were calibrated against the 1601 cm^{-1} band of polystyrene. Melting points were determined on a Thomas Hoover apparatus in open capillaries. Combustion analyses were performed by Atlantic Microlab, Inc., Atlanta, GA, and are reported as percents. Optical rotations were recorded on a Perkin-Elmer Model 214 polarimeter.

{2-[(*N*-Decyl-*N*-ethylcarbamoyl)methoxy]acetylamino} triglycyl-L-leucyldiglycyl acetic acid benzyl ester, ((10,2)DGA-GGGLGGG-OBz) 1

***N*-Decylacetamide.** To decylamine (6.5 g, 41.3 mmol) dissolved in dry CH_2Cl_2 (50 mL), acetic anhydride (3.8 g, 37.2 mmol) was added dropwise at 0 °C. The reaction was stirred at room temperature overnight. The reaction mixture was washed with dil. aq. HCl (2 × 50 mL), dried over $MgSO_4$, and evaporated. The crude product was recrystallized from hexanes to give a white solid (7.30 g, 98%) mp 50–51 °C, Lit.[16] 39–41 °C. ^{1}H-NMR: 0.87 (3H, t, J = 6.6 Hz, $CH_3(CH_2)_7CH_2CH_2N$), 1.21 (14H, pseudo-s, $CH_3(CH_2)_7CH_2CH_2N$), 1.44 (2H, m, $CH_3(CH_2)_7CH_2CH_2N$), 1.93 (3H, s, $COCH_3$), 3.16 (1H, t, J = 7.0 Hz $CH_3(CH_2)_7CH_2CH_2N$), 3.18 (1H, t, J = 7.0 Hz $CH_3(CH_2)_7CH_2CH_2N$), 5.98 (1H, bs, NH). ^{13}C-NMR: 14.0, 22.5, 23.1, 29.2, 29.4, 29.5, 31.8, 39.6, 170.1. IR ($CHCl_3$): 3284, 2925, 2916, 2871, 2850, 1641, 1566, 1466, 1380, 1298, 1171, 1110, 1001, 984, 957, 910, 852, 812, 786, 723 cm^{-1}.

***N*-Ethyl-*N*-decylamine.** To $LiAlH_4$ (1.60 g, 42.2 mmol) suspended in dry THF (40 mL), $C_{10}H_{21}NHCOCH_3$ (5.6 g, 28.4 mmol) dissolved in THF (30 mL) was added dropwise and the reaction was refluxed for 48 h. The reaction was cooled down and ethyl acetate was added to decompose excess of $LiAlH_4$. The mixture was poured into dil. aq. H_2SO_4 (250 mL) and extracted with Et_2O (2 × 100 mL). The aqueous phase was adjusted to pH 10 with NaOH pellets and the mixture was extracted with Et_2O (3 × 50 mL), dried over $MgSO_4$, and the solvent was evaporated *in vacuo*. Bulb-to-bulb distillation using a Kugelrohr apparatus (85–89 °C, 0.35 mmHg) gave the product (8.2 g, 78%) as a colorless oil. ^{1}H-NMR: 0.87 (3H, t, J = 6.6 Hz, $CH_3(CH_2)_7CH_2CH_2N$), 1.01 (1.5H, t, J = 7.2 Hz, $NHCH_2CH_3$), 1.11 (1.5H, t, J = 7.2 Hz, $NHCH_2CH_3$), 1.25 (14H, pseudo-s, $CH_3(CH_2)_7CH_2CH_2N$), 1.48 (2H, m, $CH_3(CH_2)_7CH_2CH_2N$), 1.85 (1H, bs, NH), 2.35–2.70 (4H, overlapping signals due to $CH_3(CH_2)_7CH_2CH_2N$) and CH_3CH_2N. ^{13}C-NMR (75 MHz, $CDCl_3$): 11.6, 14.1, 15.2, 22.7, 26.9, 27.4, 27.7, 29.3, 29.5, 29.6, 30.1, 31.9, 44.1, 46.9, 49.9, 53.0. IR (neat): 3310, 3263, 3093, 2959, 2925, 2854, 2811, 1720, 1645, 1574, 1466, 1379, 1298, 1204, 1134, 1069, 722 cm^{-1}.

***N*-Decyl-*N*-ethylcarbamoyl)methoxyacetic acid ((10,2)DGAOH).** A solution of $C_{10}H_{21}NHCH_2CH_3$ (7.11 g, 38.4 mmol) and diglycolic anhydride (4.45 g, 38.4 mmol) in THF (60 mL) was refluxed for 24 h. The solvent was evaporated and the crude product was dissolved in Et_2O (150 mL) and washed with 10% aqueous HCl (2×20 mL). The acid was extracted with 10% NaOH (3 ×30 mL), and the water layer was acidified with conc. HCl to pH 2, extracted with CH_2Cl_2 (4 × 30 mL), dried ($MgSO_4$) and evaporated to give the product (6.48 g, 56%) as a yellow oil, which was used directly in the next step. ^{1}H-NMR: 0.87 (3H, t, J = 6.6 Hz, $CH_3(CH_2)_7CH_2CH_2N$), 1.10–1.35 (17H, overlapping signals due to NCH_2CH_3 and $CH_3(CH_2)_7CH_2CH_2N$), 1.55 (2H, m, $CH_3(CH_2)_7CH_2CH_2N$), 3.00–3.50 (4H, m, $CH_3(CH_2)_7CH_2CH_2N$ and CH_3CH_2N), 4.21 (2H, s, $COCH_2$), 4.38 and 4.39 (2H, s, $COCH_2$). ^{13}C-NMR: 12.6, 13.8, 14.1, 14.2, 22.6, 26.8, 26.9, 27.5, 28.7, 29.2, 29.3, 29.4, 29.5, 31.8, 41.3, 41.7, 46.4, 46.6, 71.0, 71.1, 72.8, 72.9, 170.4, 171.8. IR (neat): 2927, 2855, 1742, 1619, 1461, 1439, 1378, 1216, 1135, 1047, 972, 880, 794, 722 cm^{-1}.

{2-[(*N*-Decyl-*N*-ethylcarbamoyl)methoxy]acetylamino}diglycylacetic acid benzyl ester ((10,2)DGA–GGGOCH$_2$Ph). To (10,2)DGAOH (1.43 g, 4.8 mmol) dissolved in CH_2Cl_2 (30 mL), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (0.96 g, 5.0 mmol) and HOBt (0.70 g, 5.2 mmol) were added and the mixture was stirred at room temperature. After 0.5 h, TsOH · GGG–OCH_2Ph (2.15 g, 4.8 mmol) and Et_3N (3.0 mL) were added and stirring was continued overnight. After evaporation of the solvent, the residue was dissolved in EtOAc (50 mL), washed with 5% citric acid (20 mL), water (20 mL), 5% $NaHCO_3$ (20 mL), and brine (20 mL), dried ($MgSO_4$), and evaporated. The resulting oil was chromatographed (SiO_2, 10% MeOH–CH_2Cl_2) to give the tripeptide derivative (1.94 g, 73%) as a colorless oil. ^{1}H-NMR: 0.86 (3H, t, J = 6.6 Hz,

$CH_3(CH_2)_7CH_2CH_2N$), 1.05–1.30 (17H, overlapping signals due to NCH_2CH_3 and $CH_3(CH_2)_7CH_2CH_2N$), 1.50 (2H, bs, $CH_3(CH_2)_7CH_2CH_2N$), 3.00–3.35 (4H, m, $CH_3(CH_2)_7CH_2CH_2N$ and CH_3CH_2N), 3.95–4.05 (6H, overlapping signals due to Gly $NHCH_2$), 4.09 (2H, s, $COCH_2$), 4.29 and 4.30 (2H, s, $COCH_2$), 5.13 (2H, s, $PhCH_2O$), 7.21 (1H, t, $J = 6.0$ Hz, *NH*), 7.30–7.40 (5H, m, H_{Ar}), 7.85–7.95 (1H, m, *NH*), 8.27 (1H, t, $J = 6.0$ Hz, *NH*). ^{13}C-NMR: 12.7, 13.9, 14.0, 22.6, 26.8, 27.0, 28.8, 29.2, 29.3, 29.4, 29.5, 29.6, 31.8, 41.2, 43.0, 45.9, 46.5, 67.0, 69.7, 71.7, 71.8, 128.2, 128.4, 128.6, 135.3, 168.3, 169.6, 169.7, 169.8, 169.9, 170.0, 171.3. IR ($CHCl_3$): 3302, 2926, 2855, 1749, 1656, 1541, 1457, 1358, 1260, 1193, 1129, 1032, 697 cm^{-1}.

{2-[(*N*-Decyl-*N*-ethylcarbamoyl)methoxy]acetylamino}diglycylacetic acid ((10,2)DGA–GGGOH). (10,2)DGA–GGG–OCH_2Ph (2.3 g, 3.3 mmol) was dissolved in EtOH (abs, 40 mL) and 10% Pd/C (0.13 g) was added and this mixture was shaken under 60 psi of H_2 for 3 h. The reaction mixture was heated to reflux and filtered through a celite pad. The solvent was evaporated under reduced pressure to afford a white solid (1.50 g, 96%), mp 158–159 °C. ^{1}H-NMR (CD_3OD): 0.90 (3H, t, $J = 6.6$ Hz, $CH_3(CH_2)_7CH_2CH_2N$), 1.05–1.45 (17H, overlapping signals due to NCH_2CH_3 and $CH_3(CH_2)_7CH_2CH_2N$), 1.56 (2H, bs, $CH_3(CH_2)_7CH_2CH_2N$), 3.15–3.40 (4H, m, $CH_3(CH_2)_7CH_2CH_2N$ and CH_3CH_2N), 3.90–4.00 (6H, overlapping signals due to Gly $NHCH_2$), 4.11 (2H, s, $COCH_2$), 4.38 and 4.40 (2H, s, $COCH_2$). ^{13}C-NMR (CD_3OD): 13.2, 14.2, 14.6, 23.8, 28.0, 28.2, 28.8, 29.9, 30.6, 30.7, 30.8, 33.2, 41.9, 42.2, 42.5, 43.4, 43.5, 46.9, 47.0, 70.2, 70.3, 71.7, 170.6, 170.7, 172.2, 172.3, 172.9, 173.4. IR ($CHCl_3$): 2953, 2920, 2851, 1719, 1694, 1674, 1558, 1466, 1415, 1274, 1230, 1203, 1162, 1040, 1029, 908, 732, 651 cm^{-1}.

Triglycine benzyl ester toluenesulfonic acid salt (TsOH · GGG–OCH_2Ph). GGG (3.0 g, 15.9 mmol) and *p*-toluenesulfonic acid (monohydrate, 3.6 g, 18.9 mmol) were added to a mixture of benzyl alcohol (20 mL) and toluene (30 mL). The mixture was heated to reflux and water was removed by using a Dean–Stark trap. When no more water appeared in the distillate, the mixture was cooled, diluted with Et_2O (50 mL) and cooled (0 °C) for 2 h. The crystalline *p*-toluenesulfonate of GGG–OBz was collected on a filter, washed with Et_2O (50 mL), and dried. After recrystallization from methanol–ether the salt (5.5 g, 77%) melted at 176–177 °C. ^{1}H-NMR: 2.34 (3H, s, CH_3Ph), 3.74 (2H, s, Gly NCH_2), 3.97 (4H, s, Gly NCH_2), 5.14 (2H, s, $PHCH_2O$), 7.21 (2H, d, $J = 8.4$ Hz, Tosyl H_{Ar}), 7.30–7.35 (5H, m, Ph H_{Ar}), 7.69 (2H, d, $J = 8.4$ Hz, Tosyl H_{Ar}). ^{13}C-NMR: 21.4, 41.7, 42.1, 43.2, 68.1, 127.2, 129.5, 129.6, 129.9, 130.2, 137.5, 142.1, 143.7, 168.4, 171.4, 172.2. IR (KBr): 3331, 3083, 1747, 1670, 1545, 1456, 1406, 1362, 1202, 1125, 1035, 1011, 913, 817, 736, 685 cm^{-1}.

***N*-Benzyloxycarbonyl-L-leucyldiglycyl acetic acid benzyl ester (Boc-LGGG–OCH_2Ph).** Boc-L-Leucine (0.13 g, 0.56 mmol) and TsOH · GGG–OCH_2Ph (0.25 g, 0.56 mmol) were dissolved in CH_2Cl_2 (20 mL) containing Et_3N (0.2 mL). This mixture was cooled to 5 °C and 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.118 g, 0.62 mmol) was added followed by 1-hydroxybenzotriazole (HOBt, hydrate, 0.085 g, 0.63 mmol). Reaction was stirred at room temperature overnight. The solvent was evaporated and the residue was chromatographed (SiO_2 column, 5% MeOH–$CHCl_3$) to give Boc-LGGG–OCH_2Ph as a colorless solid (0.24 g, 88%), mp 63–64 °C. ^{1}H-NMR: 0.88–0.95 (6H, m, Leu $CH(CH_3)_2$), 1.39 (9H, s, $C(CH_3)_3$), 1.45–1.70 (3H, overlapping signals due to Leu $CH_2CH(CH_3)_2$), 3.80–4.10 (7H, overlapping signals due to Leu NCH and Gly NCH_2), 5.14 (2H, s, OCH_2Ph), 5.35 (1H, d, $J = 6.3$ Hz, Leu *NH*), 7.30–7.35 (5H, m, H_{Ar}), 7.41 (1H, t, $J = 6.0$ Hz, Gly *NH*), 7.59 (1H, t, $J = 6.0$ Hz, Gly *NH*). ^{13}C-NMR: 21.7, 22.8, 24.6, 28.2, 40.8, 41.2, 42.9, 43.1, 53.6, 67.1, 80.4, 128.3, 128.5, 128.7, 135.3, 156.4, 169.7, 169.8, 170.0, 174.2. Anal. Calcd for $C_{24}H_{36}N_4O_7$: C, 58.52; H, 7.37; N, 11.37%. Found: C, 58.54; H, 7.31; N, 11.34%.

L-Leucyldiglycylacetic acid benzyl ester hydrochloride (LGGG–$OCH_2Ph \cdot HCl$). Boc-LGGG–OCH_2Ph (0.24 g, 0.48 mmol) was dissolved in 4 N HCl in dioxane (10 mL) at 5 °C and the reaction mixture was stirred for 1 h. The solvent was evaporated *in vacuo* and the residue was used in the next reaction without further purification.

(10,2)DGA–GGGLGGG–OCH_2Ph, 1. To (10,2)DGA–GGG–OH (0.22 g, 0.46 mmol) suspended in CH_2Cl_2 (20 mL), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.10 g, 0.52 mmol) and HOBt (0.07 g, 0.52 mmol) was added and reaction was stirred for 0.5 h. Then LGGG–$OCH_2Ph \cdot HCl$ (0.17 g, 0.48 mmol) in CH_2Cl_2 (20 mL) containing Et_3N (1.5 mL) was added and reaction mixture was stirred for 48 h. The mixture was evaporated and the residue washed with 5% citric acid (20 mL), water (20 mL), 5% $NaHCO_3$ (20 mL), brine (20 mL), dried ($MgSO_4$), and evaporated. The oily product was chromatographed (SiO_2, 10% MeOH–CH_2Cl_2) to give **1** (0.39 g, 70%) as a white solid, mp 179–81 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD ~9 : 1 v/v): 0.80 (9H, m, $CH_3(CH_2)_7CH_2CH_2N$, $CH(CH_3)_2$), 1.00–1.24 (17H, overlapping signals due to NCH_2CH_3 and $CH_3(CH_2)_7CH_2CH_2N$), 1.46 (2H, bs, $CH_3(CH_2)_7CH_2CH_2N$), 1.56 (3H, m, $CH_2CH(CH_3)_2$), 3.00–3.30 (4H, overlapping signals due to $CH_3(CH_2)_7CH_2CH_2N$ and CH_3CH_2N), 3.70–4.00 (14H, Gly NCH_2, $COCH_2O$), 4.21 (3H, overlapping signals due to Leu NCH and $COCH_2O$), 5.06 (2H, s, CH_2OPh), 7.24 (5H, m, H_{Ar}). ^{13}C-NMR ($CDCl_3$–CD_3OD ~9 : 1 v/v): 12.1, 13.2, 13.4, 20.8, 22.2, 22.3, 24.4, 26.4, 26.6, 27.2, 27.4, 28.4, 28.9, 28.96, 29.0, 29.1, 29.2, 31.5, 39.6, 40.7, 40.8, 41.0, 42.0, 42.2, 42.5, 42.6, 42.8, 45.5, 46.3, 52.5, 66.7, 68.5, 68.6, 70.3, 70.4, 127.8, 128.0, 128.2, 135.1, 168.38, 168.42, 169.5, 170.38, 170.43, 170.6, 171.0, 171.4, 174.0. Anal. Calcd for $C_{40}H_{62}N_8O_{11} \cdot 1/2\ H_2O$: C, 57.53; H, 7.89; N, 13.08%. Found: C, 57.47; H, 7.81; N, 13.10%.

{2-[(*N*,*N*-Didecylcarbamoyl)methoxy]acetylamino} triglycyl-L-leucyldiglycylacetic acid benzyl ester, (10_2DGA–GGGLGGG–OCH_2Ph) 2.

{2-[(*N*,*N*-Didecylcarbamoyl)methoxy]acetylamino}diglycylacetic acid (10_2DGA-GGG-OH) was prepared as previously described.[10]

10_2DGA–GGGLGGG–OCH_2Ph, 2. 10_2DGA–GGG–OH (0.21 g, 0.45 mmol) was suspended in CH_2Cl_2 (50 mL) and cooled to 0 °C. 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.10 g, 0.52 mmol), HOBt (0.08 g, 0.59 mmol), Leu–GlyGlyGly–$OCH_2Ph \cdot HCl$ (0.19 g, 0.45 mmol) and Et_3N (0.7 mL) were added, the mixture was stirred for 0.5 h, and stirred for 48 h at room temperature. After evaporation of the solvent, the residue was washed with 5% citric acid, 5% $NaHCO_3$, and water. The residue was chromatographed (SiO_2, $CHCl_3$–CH_3OH 95 : 5 → 9 : 1) to give a white solid. Crystallization from MeOH gave **1** (0.12 g, 28%), mp 186–188 °C. ^{1}H-NMR ($CDCl_3$–CD_3OD 9 : 1 v/v): 0.68 (12H, m, CH_3), 1.05 (28H, m, $CH_3(CH_2)_7CH_2CH_2N$), 1.32 (4H, m, $CH_3(CH_2)_7CH_2CH_2N$), 1.46 (3H, m, Leu $CH_2CH(CH_3)_2$), 2.89 (2H, t, $J = 7.5$ Hz, $CH_3CH_2(CH_2)_7CH_2N$), 3.09 (2H, t, $J = 7.5$ Hz, $CH_3CH_2(CH_2)_7CH_2N$), 3.57–3.91 (14H, overlapping signals due to Gly CH_2 and $COCH_2O$), 4.07 (2H, s, $COCH_2O$), 4.10 (1H, m, Leu *CH*), 4.94 (2H, s, OCH_2Ph), 7.14 (5H, m, H_{Ar}). ^{13}C-NMR ($CDCl_3$–CD_3OD 9 : 1 v/v): 13.9, 21.2, 22.5, 22.7, 24.7, 26.8, 26.9, 27.4, 28.6, 29.17, 29.2, 29.3,

29.4, 29.5, 31.8, 39.6, 41.0, 42.4, 42.6, 42.8, 43.0, 43.2, 46.3, 46.8, 52.7, 67.0, 68.7, 70.7, 128.1, 128.4, 128.5, 135.2, 168.5, 169.9, 170.5, 170.9, 171.1, 171.2, 171.5, 174.1. Elem. Analysis Calcd for $C_{49}H_{82}O_{11}N_8 \cdot 1/2\ H_2O$: C 60.79, H 8.64, N 11.57%. Found: C 60.85, H 8.60, N 11.46%.

{2-[(*N*,*N*-Didecylcarbamoyl)methoxy]acetylamino} triglycyl-L-azetidinyldiglycylacetic acid benzyl ester, (10_2DGA-$(Gly)_3$Aze$(Gly)_3$–OCH_2Ph) 3

***N*-Benzyloxycarbonyl-L-azetidinyldiglycylacetic acid benzyl ester (Boc–Aze–GGG–OCH_2Ph).** A solution of Boc-L-azetidine-2-carboxylic acid (0.25 g, 1.24 mmol) and GGG–OCH_2Ph tosylate (0.56 g, 1.24 mmol), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.27 g, 1.41 mmol), HOBt (0.19 g, 1.41 mmol) and Et_3N (0.6 mL) in CH_2Cl_2 (40 mL) was stirred at 5 °C for 1 h and 25 °C for 3 days. The solvent was evaporated and the residue was chromatographed (SiO_2, CH_2Cl_2–MeOH 97 : 3 → 95 : 5) to give the ester as a deliquescent solid (0.52 g, 90%). ^{1}H-NMR: 1.39 (9H, s, $C(CH_3)_3$), 2.32 (2H, m, Aze NCH_2CH_2CH), 3.85 (2H, m, Aze NCH_2CH_2CH), 3.95–4.01 (6H, overlapping signals due to Gly CH_2), 4.62 (1H, t, $J = 7.8$ Hz, NCH_2CH_2CH), 5.10 (2H, s, OCH_2Ph), 7.30 (5H, H_{Ar}), 7.42 (1H, t, $J = 5.4$ Hz, Gly *NH*), 7.62 (1H, bs, Gly *NH*), 7.82 (2H, bs, Gly *NH*). ^{13}C-NMR: 19.9, 28.4, 41.3, 42.9, 43.1, 47.8, 62.0, 67.2, 77.4, 81.2, 128.4, 128.5, 128.7, 135.3, 169.8, 169.9, 172.6.

10_2DGA–GGGAzeGGG–OCH_2Ph, 3. 10_2DGA–GGG–OH (0.51 g, 0.87 mmol) was suspended in CH_2Cl_2 (50 mL) and cooled to 0 °C. 1-(3-Dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.20 g, 1.04 mmol), HOBt (0.14 g, 1.04 mmol), Aze–GGG–OBz · HCl (0.35 g, 0.88 mmol) and Et_3N (0.8 mL) were added and the mixture was stirred for 0.5 h at 0 °C and 48 h at 25 °C. The solvent was removed and the residue chromatographed (silica, $CHCl_3$–CH_3OH 98 : 2 → 9 : 1) to give a white solid that was crystallized from methanol to give **3** (0.08 g, 10%), mp 117–119 °C. ^{1}H-NMR: 0.79 (6H, m, CH_3), 1.17 (28H, m, $CH_3(CH_2)_7CH_2CH_2N$), 1.42 (4H, m, $CH_3(CH_2)_7CH_2CH_2N$), 2.38 (2H, m, Aze NCH_2CH_2CH), 2.99 (2H, t, $J = 7.8$ Hz, $CH_3CH_2(CH_2)_7CH_2N$), 3.17 (2H, t, $J = 7.5$ Hz, $CH_3CH_2(CH_2)_7CH_2N$), 3.55–4.10 (16H, overlapping signals due to Aze NCH_2CH_2, Gly CH_2, and $COCH_2O$), 4.19 (2H, s, $COCH_2O$), 4.73 (1H, m, Aze *CH*), 5.05 (2H, m, OCH_2Ph), 7.25 (5H, m, H_{Ar}), 7.50 (2H, m, Gly *NH*), 7.69 (1H, t, $J = 5.4$ Hz, Gly *NH*), 7.83 (1H, t, $J = 5.4$ Hz, Gly *NH*), 8.19 (1H, t, $J = 5.7$ Hz, Gly *NH*), 8.28 (1H, t, $J = 5.7$ Hz, Gly *NH*). ^{13}C-NMR: 14.2, 19.8, 22.8, 27.0, 27.2, 27.8, 29.0, 29.4, 29.5, 29.7, 32.0, 39.5, 41.4, 43.0, 43.5, 46.4, 47.0, 49.0, 61.9, 67.2, 69.7, 71.6, 128.4, 128.5, 128.7, 135.5, 168.5, 170.0, 170.1, 170.2, 170.3, 170.5, 170.5, 171.2, 173.4. Elem. Analysis Calcd for $C_{47}H_{76}O_{11}N_8 \cdot 1.5H_2O$: C 59.04, H 8.33, N 11.71%. Found: C 59.07, H 8.37, N 11.62%.

{2-[(*N*-Decyl-*N*-ethylcarbamoyl)methoxy]acetylamino} triglycyl-L-prolyldiglycylacetic acid benzyl ester, ((10,2)DGA-GGGPGGG–OCH_2Ph) 4

L-Prolyldiglycyl acetic acid benzyl ester hydrochloride (PGGG-$OCH_2Ph \cdot HCl$) was prepared as previously described.[10]

(10,2)DGA–GGGPGGG–OCH_2Ph, 4

To (10,2)DGA-GGG-OH (0.25 g, 0.52 mmol) suspended in CH_2Cl_2 (20 mL), 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.11 g, 0.57 mmol) and HOBt (0.08 g, 0.59 mmol) was added and reaction was stirred for 0.5 h. Then PGGG–$OCH_2Ph \cdot HCl$ (0.22 g, 0.52 mmol) in CH_2Cl_2 (20 mL) containing Et_3N (0.15 mL) was added and reaction mixture was stirred for 48 h at 25 °C. After evaporation, the residue was washed with 5% citric acid (20 mL), water (20 mL), 5% $NaHCO_3$ (20 mL), brine (20 mL), dried ($MgSO_4$), and evaporated. The residue was chromatographed (SiO_2, 10% MeOH-CH_2Cl_2) to give **4** (0.32 g, 75%) as a white solid, mp 195 °C decomposition. $[\alpha]_D^{23}$ −17.7 deg cm^3 g^{-1} dm^{-1} (*c* 1.325, MeOH). ^{1}H-NMR: 0.87 (3H, t, $J = 6.6$ Hz, $CH_3(CH_2)_7CH_2CH_2N$), 1.05–1.35 (17H, overlapping signals due to NCH_2CH_3 and $CH_3(CH_2)_7CH_2CH_2N$), 1.51 (2H, bs, $CH_3(CH_2)_7CH_2CH_2N$), 1.95–2.15 (4H, m, Pro $NCH_2CH_2CH_2$), 3.00–3.40 (4H, m, $CH_3(CH_2)_7CH_2CH_2N$ and CH_3CH_2N), 3.40–3.55 (2H, m, Pro $NCH_2CH_2CH_2$), 3.70–4.40 (17H, overlapping signals due to Gly NCH_2, Pro *NCH* and $COCH_2O$), 5.05–5.20 (2H, m, OCH_2Ph), 7.30–7.40 (5H, m, H_{Ar}), 7.45–7.55 (2H, m, Gly *NH*), 7.85–8.00 (2H, m, Gly *NH*), 8.12 (1H, t, $J = 6.0$ Hz, Gly *NH*), 8.30–8.40 (1H, bs, Gly *NH*). ^{13}C-NMR: 13.0, 14.2, 14.3, 22.8, 25.3, 27.1, 27.3, 27.9, 29.2, 29.5, 29.6, 29.7, 32.1, 41.3, 41.5, 42.1, 42.3, 43.1, 43.2, 43.7, 46.0, 46.8, 47.1, 61.4, 67.3, 69.8, 71.6, 71.7, 128.5, 128.6, 128.8, 135.6, 168.5, 169.0, 170.3, 170.4, 170.5, 171.0, 171.1, 171.3, 173.6, 173.7. Anal. Calcd for $C_{40}H_{62}N_8O_{11}$: C, 57.82; H, 7.52; N, 13.48%. Found: C, 57.61; H, 7.56; N, 13.66%.

{2-[(*N*,*N*-Didecylcarbamoyl)methoxy]acetylamino}triglycyl-L-prolyldiglycylacetic acid benzyl ester, (10_2DGA-GGGPGGG–OBz) **5** and {2-[(*N*,*N*-didecylcarbamoyl)methoxy]acetylamino}triglycyl-L-pipecolyldiglycylacetic acid benzyl ester, (10_2DGA–GGGPipGGG–OBz) **6** were previously reported.[10]

{2-[(*N*,*N*-Didecylcarbamoyl)methoxy]acetylamino} triglycyl-*m*-aminobenzoyldiglycylacetic acid benzyl ester, (10_2DGA–GGGAbaGGG–OBz) 7

***N*-Benzyloxycarbonyl-3-aminobenzoic acid (Boc-Aba).** 3-Aminobenzoic acid (2.47 g, 18.01 mmol) and di-*tert*-butyl dicarbonate (5.30 g, 24.28 mmol) were dissolved in dioxane (18 mL) and 2 M KOH (18 mL) and the mixture was stirred for 20 h at room temperature. The dioxane was evaporated and the remaining solution was diluted with 1 M KOH (35 mL) and washed twice with ethyl ether; the water phase was then acidified with conc. HCl and a brown solid precipitated from the solution. The crude product was filtrated, washed with water and then dissolved in ethyl acetate and washed twice with water; the solvent was removed and the remaining solid recrystallized in the freezer from CH_2Cl_2–MeOH (just enough methanol to dissolve all the solid) and then triturated with hexanes to give a white solid (3.40 g, 80%), mp 195–197 °C. ^{1}H-NMR (DMSO-d_6): 1.46 (9H, s, $C(CH_3)_3$), 7.34 (1H, t, $J = 8.1$ Hz, H_{Ar}), 7.52 (1H, d, $J = 6.3$ Hz, H_{Ar}), 7.60 (1H, d, $J = 7.8$ Hz, H_{Ar}), 8.13 (1H, s, H_{Ar}), 9.54 (1H, s, Ar*NH*), 12.88 (1H, bs, *COOH*). ^{13}C-NMR (DMSO-d_6): 28.1, 79.3, 118.8, 122.3, 122.9, 128.8, 131.3, 139.8, 152.8, 167.3.

***N*-Benzyloxycarbonyl-aminobenzoyldiglycyl acetic acid benzyl ester (Boc–AbaGGG–OBz).** Boc-Aba (0.31 g, 1.31 mmol) was suspended in $CHCl_3$ (20 mL) and added to 1-(3-dimethylaminopropyl)-3-ethyl carbodiimide hydrochloride (0.26 g, 1.36 mmol). The mixture was stirred for 0.5 h then TsOH · GGG–OBz (0.59 g, 1.31 mmol) in $CHCl_3$ (10 mL) and Et_3N (0.3 mL) was added and the reaction stirred for 48 h at room temperature. The solvent was then evaporated and the residue purified by column chromatography (silica gel, $CHCl_3$–CH_3OH 95 : 5 → 90 : 10) to give a white solid (0.20 g, 83%), mp 135–137 °C. ^{1}H-NMR: 1.45 (9H, s, $C(CH_3)_3$), 3.91 (2H, d, $J = 5.1$ Hz, Gly CH_2), 3.97 (2H, d, $J = 5.7$ Hz, Gly CH_2), 4.02 (2H, d, $J = 5.4$ Hz, Gly CH_2), 5.04 (2H, s, OCH_2Ph), 7.23 (8H, overlapping signals due to H_{Ar} and *NH*), 7.39 (1H, d, $J = 7.8$ Hz, H_{Ar}), 7.65 (1H, bs, H_{Ar}), 7.71 (1H, bm, *NH*), 7.90 (1H, bt, *NH*), 7.97 (1H, bt, *NH*). ^{13}C-NMR: 28.4, 41.4, 43.0, 43.9, 67.3, 80.8, 117.7, 121.6, 122.2, 128.4, 128.6, 128.7, 129.3, 133.9, 135.3, 139.3,

153.3, 168.4, 170.2, 170.3, 170.7. Anal. Calcd for $C_{25}H_{30}N_4O_7 \cdot 1/2\ H_2O$: C, 59.16; H, 6.16; N, 11.03%. Found: C, 59.42; H, 6.12; N, 10.96%.

10_2DGA–GGGAbaGGG–OBz, 7

Boc–AbaGGG–OBz (0.30 g, 0.60 mmol) was dissolved in dioxane (5 mL) and cooled to 0 °C then a 4 N HCl solution in dioxane (5.0 mL, 20 mmol) was added and the resulting solution was stirred at room temperature under nitrogen atmosphere for 3 hours; the solvent was removed and the product carefully dried and used right away for the next reaction. This compound, HCl · 3-aminobenzoic acid-GGG–OBz (0.26 g, 0.60 mmol) was mixed with 10_2DGA–GGG–OH (0.42 g, 0.72 mmol), PyCloP (0.30 g, 0.72 mmol) and CH_2Cl_2 (35 mL). The mixture was cooled to 0 °C then diisopropylethylamine (0.26 mL, 2.01 mmol) was added and the resulting solution was stirred for 1 h. The reaction was then continued at room temperature for 4 days. The solvent was removed and the product purified by column chromatography (silica gel, $CHCl_3$–CH_3OH 95 : 5 → 8 : 2) to give a white solid (0.20 g, 35%). Mp: dec. ^{1}H-NMR (DMSO-d_6): 0.83 (6H, t, J = 5.4 Hz, CH_3), 1.21 (28H, m, $NCH_2CH_2(CH_2)_7CH_3$), 1.43 (4H, bs, $NCH_2CH_2(CH_2)_7CH_3$), 3.09 (2H, t, J = 7.5 Hz, $NCH_2CH_2(CH_2)_7CH_3$), 3.17 (2H, t, J = 7.5 Hz, $NCH_2CH_2(CH_2)_7CH_3$), 3.75–3.90 (12H, overlapping signals due to Gly CH_2), 3.97 (2H, s, $COCH_2O$), 4.26 (2H, s, $COCH_2O$), 5.11 (2H, s, OCH_2Ph), 7.34 (6H, m, H_{Ar}), 7.56 (1H, d, J = 7.5 Hz, H_{Ar}), 7.78 (1H, d, J = 7.5 Hz, H_{Ar}), 8.07 (1H, s, H_{Ar}), 8.25 (5H, m, NH), 8.71 (1H, s, NH), 9.98 (1H, s, ArNH). ^{13}C-NMR (DMSO-d_6): 13.9, 22.1, 26.2, 26.4, 27.1, 28.3, 28.7, 28.8, 29.0, 31.3, 40.7, 41.7, 41.8, 42.2, 42.7, 42.8, 45.1, 46.1, 65.9, 68.8, 70.3, 118.7, 122.0, 122.1, 127.9, 128.0, 128.1, 128.6, 134.7, 135.9, 138.9, 166.5, 167.8, 168.1, 169.26, 169.32, 169.4, 169.5, 169.59, 169.62. Anal. Calcd for $C_{50}H_{76}N_8O_{11} \cdot 2\ H_2O$: C, 59.98; H, 8.05; N, 11.19%. Found: C, 59.66; H, 7.71; N, 10.80%.

Formation of vesicles

Liposomes were prepared from 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dioleoyl-*sn*-glycero-3-phosphate (DOPA), both obtained from Avanti Polar Lipids as $CHCl_3$ solutions (vials with 100 mg in 4.0 mL). Dry lipid films of DOPC–DOPA (20 mg, 7 : 3 w/w) were dissolved in Et_2O (0.5 mL) and then 0.5 mL of internal buffer (600 mM KCl : 10 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), pH adjusted to 7.0) were added. Sonication of the mixture for ~10 s gave an opalescent dispersion. Evaporation under mild vacuum (30 °C) removed the organic solvent and the remaining suspension was filtered (200 nm filter membrane, 5×, using a mini extruder) and then passed through a Sephadex G25 column (previously equilibrated with external buffer (400 mM K_2SO_4 : 10 mM HEPES, pH adjusted to 7.0). Analysis by laser light scattering (Coulter N4MD submicron particle analyzer) showed that the vesicular diameter was consistently ~200 nm. The final lipid concentration was assessed through colorimetric determination of the phospholipid–ammonium ferrothiocyanate complex.[17]

Chloride release from liposomes

A chloride selective electrode (Accumet Chloride Combination Electrode) was used to assay ~200 nm, 0.31 mM phospholipid vesicles. The electrode was inserted in the vesicle suspension (2 mL), equilibrated, the voltage output was recorded, and after five minutes, aliquots of the ionophore (~9 mM in 2-propanol) were added. The amount of 2-propanol added was limited to ≤20 μL to avoid a solvent effect on the liposomes. Liposomal lysis was induced by 2% aqueous Triton X-100 (100 μL) and the data collected were normalized to the value obtained after lysis. The data were collected using a DigiData 1322A series interface and Axoscope 9.0 software.

Carboxyfluorescein dequenching from unilamellar liposomes

The reverse phase procedure of Szoka and Papahadjopoulos[18] was used to prepare the liposomes used in this study. A mixture (10 mg) composed of 30% (w/w) 1,2-dioleoyl-*sn*-glycero-3-phosphate and 70% 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, (Avanti Polar Lipids, AL) was dissolved in 0.5 mL of Et_2O. To this was added 0.5 mL of 20 mM carboxyfluorescein in 100 mM KCl : 10 mM HEPES (pH = 7.0) and the final pH was adjusted to 7.0 with KOH. This mixture was sonicated at 1200 W for 3 × 20 s at 20 °C to produce a stable emulsion. The Et_2O was removed (water aspirator, 15 °C) in a round bottom flask rotating at 40 rpm. The 0.5 mL suspension was supplemented with an additional 0.5 mL of 20 mM carboxyfluorescein solution. This mixture was passed (five times) through a 200 nm nucleopore filter. The extra-vesicular carboxyfluorescein was removed by passing the liposome–dye mixture over a 1 × 20 cm Sephacryl G-25 column (Sigma) in 100 mM KCl : 10 mM HEPES (pH = 7.0). The liposome peak was collected and analyzed by dynamic light scattering, diameter = 182 ± 12 nm. Phospholipid concentration in this fraction was determined to be 3.3 mg ml^{-1}.[17]

Carboxyfluorescein-containing vesicles were diluted to 0.66 μg ml^{-1} in a reaction volume of 500 μL. The fluorescence (excitation 497 nm: emission 520 nm at 2 nm bandpass) was monitored at 25 °C. Compounds were added as a 5 mM solution in 2-propanol with mixing to the indicated concentrations. Data were digitized at 10 points per second and subsequently decimated to one point per second. Dequenching, F_{520}, was computed as the fraction of total release upon addition of 1% Triton X-100:

$$F_{520} = \frac{F - F_0}{F_{\text{Triton}} - F_0} \qquad (1)$$

where F_0 is the fluorescence at zero time and F_{Triton} is the fluorescence after Triton X-100 treatment. Dequenching data were fit using a nonlinear least squares method based upon the Levenberg–Marquardt algorithm, which generated c^2 values indicating the goodness of fit, for each group of averaged data sets. The number of individual trials generating the data set (degrees of freedom) was used to obtain the p value for the individual fits and kinetic constants. In all cases, the c^2 values for individual fits were less than 0.05 and the resulting p values varied from 0.05 to 0.001 depending upon the number of trials. Data were fit to the equation,

$$F_{520} = F_0 + A_1(1 - e^{\text{time}/\tau}) + m \cdot \text{time} \qquad (2)$$

as described previously.[19] In this equation, F_0 is the fluorescence at time zero, A_1 is the size of the exponential component, τ is the time constant for pore activation, and m is the slope of the linear dequenching resulting from multiple pores per vesicle.

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