

# The Depth of Molecular Recognition: Voltage-Sensitive Blockage of Synthetic Multifunctional Pores with Refined Architecture

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## Supporting Information

**General.** As in (S1), Supporting Information. ANTS and DPX were purchased from Molecular Probes, valinomycin, melittin, ADP, ATP, phytate and safranin O from Sigma or Fluka-Aldrich.

**Abbreviations.** ANTS: 8-Aminonaphthalene-1,3,6-trisulfonate; Arg, R: *L*-Arginine; Asn, N: *L*-Asparagine; BLM: Bilayer lipid membrane; DMF: *N,N*-Dimethylformamide; DPX: *p*-Xylenebis(pyridinium)bromide; EDC: 1-[3-(Dimethylamino)propyl]-3-ethylcarbodiimide, EYPC-LUVs: Egg yolk phosphatidylcholine large unilamellar vesicles; Fmoc: 9-Fluorenylmethoxycarbonyl; *Gla*: -OCH<sub>2</sub>CO- (*H-Gla-OH*: glycolic acid); HATU: *N*-[(dimethylamino)-1*H*-1,2,3-triazolo[4,5-*b*]pyridin-1-ylmethylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; HBTU: *N*-[(1*H*-benzotriazol-1-yl)(dimethylamino)methylene]-*N*-methylmethanaminium hexafluorophosphate *N*-oxide; His, H: *L*-Histidine; HOBt: 1-Hydroxybenzotriazole; Leu, L: *L*-Leucine; MES: 2-Morpholinoethanesulfonic acid monohydrate; Pmc: 2,2,5,7,8,-Pentamethylchromane-6-sulfonyl; TEA: Triethylamine; TES: *N*-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid; TFA: Trifluoroacetic acid; Trt: Trityl.

**Fmoc-Asn(Trt)-Leu-NH<sub>2</sub>, general procedure A.** EDC•HCl (288 mg, 1.5 mmol), HOBt (200 mg, 1.3 mmol), and H-Leu-NH<sub>2</sub> (166 mg, 1.0 mmol) and TEA (840 ml, 6.0 mmol) were added to a solution of Fmoc-Asn(Trt)-OH (657 mg, 1.1 mmol) in CH<sub>2</sub>Cl<sub>2</sub> (8 ml) at 0 °C. After stirring for 4 hours in the dark at rt, the reaction mixture was diluted with CH<sub>2</sub>Cl<sub>2</sub>, washed successively with saturated aqueous NaHCO<sub>3</sub>, brine, 1 M aqueous KHSO<sub>4</sub>, and brine, dried over anhydrous Na<sub>2</sub>SO<sub>4</sub>, and concentrated in vacuo. Purification of the crude product by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 50:1) yielded pure Fmoc-Asn(Trt)-Leu-NH<sub>2</sub> (479 mg, 68%) as colorless solid. TLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 20:1): *R<sub>f</sub>* 0.33; [α]<sub>D</sub><sup>20</sup> = -22.7 (c = 0.77, CH<sub>2</sub>Cl<sub>2</sub>/MeOH 1:1); mp: 203-204 °C; IR: ν 3286 (m), 2958 (w), 1689 (s), 1652 (s), 1530 (s), 1448 (s), 1299(m), 1235 (m), 1038 (m), 739 (s), 696 (s); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD): δ 7.70 (d, <sup>3</sup>*J* (H,H) = 7.3 Hz, 2H), 7.64 (d, <sup>3</sup>*J* (H,H) = 7.3 Hz, 2H), 7.39 (t, <sup>3</sup>*J* (H,H) = 7.3 Hz, 2H), 7.29-7.17 (m, 17H), 4.46 (dd, <sup>3</sup>*J* (H,H) = 7.7 Hz, <sup>3</sup>*J* (H,H) = 6.4 Hz, 1H), 4.42 (d, <sup>3</sup>*J* (H,H) = 6.8 Hz, 2H), 4.33 (dd, <sup>3</sup>*J* (H,H) = 7.0 Hz, <sup>3</sup>*J* (H,H) = 5.0 Hz, 1H), 4.23 (t, <sup>3</sup>*J* (H,H) = 6.8 Hz, 1H), 2.89 (dd, <sup>2</sup>*J* (H,H) = 15.4 Hz, <sup>3</sup>*J* (H,H) = 6.4 Hz, 1H), 2.75 (dd, <sup>2</sup>*J* (H,H) = 15.4 Hz, <sup>3</sup>*J* (H,H) = 7.7 Hz, 1H), 1.63-1.43 (m, 3H), 0.88 (d, <sup>3</sup>*J* (H,H) = 6.2 Hz, 3H), 0.85 (d, <sup>3</sup>*J*

(H,H) = 6.0 Hz, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  173.7 (s), 171.7 (s), 156.1 (s), 145.9 (s), 145.3 (s), 142.6 (s), 130.1 (d), 128.8 (d), 128.7(d), 128.2 (d), 127.8 (d), 126.2 (d), 121.0 (d), 71.8 (s), 67.9 (t), 53.0 (d), 52.7 (d), 48.0 (d), 41.4 (t), 39.1 (t), 25.6 (d), 23.3 (q), 21.4 (q); ESI-MS ( $\text{MeOH}/\text{CH}_2\text{Cl}_2$  1:1):  $m/z$  731 (100,  $[\text{M} + \text{Na}]^+$ ).

**H-Asn(Trt)-Leu-NH<sub>2</sub>**, *general procedure B*. A solution of Fmoc-Asn(Trt)-Leu-NH<sub>2</sub> (1.76 g, 2.48 mmol) in 8 ml DMF containing 5% piperidine was stirred for 25 min at rt. Concentration *in vacuo* and purification of the crude product by column chromatography ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  20:1, then 7:1) yielded pure H-Asn(Trt)-Leu-NH<sub>2</sub> (1.19 g, 98%) as colorless powder. TLC ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  10:1):  $R_f$  0.30;  $[\alpha]_D^{20} = -30.3$  ( $c=1.01$ ,  $\text{CH}_2\text{Cl}_2$ ); mp = 102-104 °C; IR:  $\nu$  3404 (m), 3350 (m), 2961 (m), 1683 (s), 1594 (w), 1492 (s), 1204 (w), 1018 (m), 810 (m);  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.22-7.19 (m, 15H), 4.33 (dd,  $^3J$  (H,H) = 10.0 Hz,  $^3J$  (H,H) = 4.9 Hz, 1H), 3.63 (dd,  $^3J$  (H,H) = 7.3 Hz,  $^3J$  (H,H) = 5.8 Hz, 1H), 2.78 (dd,  $^2J$  (H,H) = 15.7 Hz,  $^3J$  (H,H) = 5.8 Hz, 1H), 2.63 (dd,  $^2J$  (H,H) = 15.7 Hz,  $^3J$  (H,H) = 7.3 Hz, 1H), 1.61-1.45 (m, 3H), 0.94 (d,  $^3J$  (H,H) = 6.6 Hz, 3H), 0.90 (d,  $^3J$  (H,H) = 6.3 Hz, 3H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  177.6 (s), 176.6 (s), 172.7 (s), 146.0 (s), 130.0 (d), 128.7 (d), 127.9 (d), 71.6 (s), 53.1 (d), 52.8 (s), 42.1 (t), 42.0 (t), 25.9 (d), 23.5 (q), 21.8 (q); ESI-MS ( $\text{CH}_2\text{Cl}_2$ ):  $m/z$  487 (100,  $[\text{M} + \text{H}]^+$ ).

**Z-Leu-Asn(Trt)-Leu-NH<sub>2</sub>**. Coupling of H-Asn(Trt)-Leu-NH<sub>2</sub> (582 mg, 1.20 mmol) with Z-Leu-OH (433 mg, 1.63 mmol) following the *general procedure A* and purification of the crude product by column chromatography ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  30:1) yielded pure Z-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (800 mg, 89%) as colorless powder. TLC ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  15:1):  $R_f$  0.31;  $[\alpha]_D^{20} = -42.9$  ( $c = 0.99$ ,  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  1:1); mp > 230 °C; IR:  $\nu$  3289 (m), 2957 (m), 1658 (s), 1492 (s), 1448 (w), 1234 (m), 1038 (m), 740 (m), 697 (m);  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.27-7.16 (m, 20H), 5.05 (d,  $^2J$  (H,H) = 12.4 Hz, 1H), 4.98 (d,  $^2J$  (H,H) = 12.4 Hz, 1H), 4.57 (dd,  $^3J$  (H,H) = 7.6 Hz,  $^3J$  (H,H) = 5.8 Hz, 1H), 4.25 (dd,  $^3J$  (H,H) = 10.6 Hz,  $^3J$  (H,H) = 4.0 Hz, 1H), 4.07 (dd,  $^3J$  (H,H) = 7.6 Hz,  $^3J$  (H,H) = 7.6 Hz, 1H), 2.92 (dd,  $^2J$  (H,H) = 15.8 Hz,  $^3J$  (H,H) = 7.6 Hz, 1H), 2.75 (dd,  $^2J$  (H,H) = 15.8 Hz,  $^3J$  (H,H) = 5.8 Hz, 1H), 1.59-1.48 (m, 6H), 0.89-0.80 (m, 12H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  176.9 (s), 174.7 (s), 172.2 (s), 171.1 (s), 157.9 (s), 145.0 (s), 137.0 (s), 129.4 (d), 129.0 (d), 128.6 (d), 128.3 (d), 128.2 (d), 127.4 (d), 71.2 (s), 67.5 (t), 54.6 (d), 52.6 (d), 51.0 (d), 41.5 (t), 40.6 (t), 37.9 (t), 25.3 (d), 23.5 (q), 23.2 (q), 21.8 (q), 21.3 (q); ESI-MS ( $\text{MeOH}/\text{CH}_2\text{Cl}_2$  1/1):  $m/z$  756 (100,  $[\text{M} + \text{Na}]^+$ ).

**H-Leu-Asn(Trt)-Leu-NH<sub>2</sub>**, *general procedure C*.  $\text{Pd}(\text{OH})_2/\text{C}$  (25 mg) was added to a solution of Z-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (800 mg, 1.09 mmol) in  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  1:1 (16 ml). The suspension was degassed and set under a  $\text{H}_2$  atmosphere. After stirring for 4 h, the reaction mixture was filtered through celite and aluminum oxide (basic), and the filtrate was concentrated *in vacuo*. Column chromatography ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  30:1, then 7:1) yielded pure H-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (442.0 g, 68%) as colorless powder. TLC ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  10:1):  $R_f$  0.26;  $[\alpha]_D^{20} = -30.0$  ( $c=0.7$ ,  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  1:1); mp > 230 °C; IR:  $\nu$  3268 (m), 2957 (m), 1655 (s), 1636 (s), 1528 (s), 1444 (m), 1303 (m), 901 (w), 751 (w), 696 (s);  $^1\text{H}$  NMR (500 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.28-7.19 (m, 15H), 4.67 (dd,  $^3J$  (H,H) = 7.8 Hz,  $^3J$  (H,H) = 6.3 Hz, 1H), 4.30 (dd,  $^3J$  (H,H) = 10.9 Hz,  $^3J$  (H,H) = 4.0 Hz, 1H), 3.33 (dd, 1H), 2.97 (dd,  $^2J$  (H,H) = 15.8 Hz,  $^3J$  (H,H) = 7.8 Hz, 1H), 2.79 (dd,  $^2J$  (H,H) = 15.8 Hz,  $^3J$  (H,H) = 6.3 Hz, 1H), 1.71-1.47 (m, 4H), 1.37-1.30 (m, 2H), 0.94-0.86 (m, 12H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  177.9 (s), 177.6 (s), 173.2 (s), 171.8 (s), 145.9 (s), 130.1 (d), 128.7 (d), 127.8 (d), 71.7 (s), 54.3 (d), 52.9

(d), 51.2 (d), 45.4 (t), 41.5 (t), 38.9 (t), 25.8 (d), 25.7 (d), 23.7 (q), 23.4 (q), 22.5 (q), 21.5 (q); ESI-MS (CH<sub>2</sub>Cl<sub>2</sub>): m/z 1222 (100, [2M + Na]<sup>+</sup>), 622 (40, [M + Na]<sup>+</sup>), 600 (25, [M + H]<sup>+</sup>).

**Fmoc-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub>.** Coupling of H-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (516 mg, 0.86 mmol) with Fmoc-Asn(Trt)-OH (693 mg, 1.16 mmol) following the *general procedure A* and purification of the crude product by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 40:1) yielded pure Fmoc-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (801 mg, 79%) as colorless powder. TLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 15:1): R<sub>f</sub> 0.44; [α]<sup>20</sup><sub>D</sub> = -21.2 (c = 0.66, CH<sub>2</sub>Cl<sub>2</sub>/MeOH 20:1); mp > 230 °C; IR: ν 3276 (m), 1657 (s), 1633 (s), 1490 (s), 1446 (m), 1225 (m), 1036 (m), 740 (m), 698 (m); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD/CDCl<sub>3</sub> 1:4): δ 7.81 (d, <sup>3</sup>J (H,H) = 7.5 Hz, 2H), 7.61 (d, <sup>3</sup>J (H,H) = 7.5 Hz, 2H), 7.44 (t, <sup>3</sup>J (H,H) = 7.5 Hz, 2H), 7.34 (t, <sup>3</sup>J (H,H) = 7.5 Hz, 2H), 7.30-7.10 (m, 30H), 4.55 (dd, <sup>3</sup>J (H,H) = 7.6 Hz, <sup>3</sup>J (H,H) = 4.6 Hz, 1H), 4.49-4.34 (m, 4H), 4.23 (t, <sup>3</sup>J (H,H) = 6.8 Hz, 1H), 4.10 (dd, <sup>3</sup>J (H,H) = 9.1 Hz, <sup>3</sup>J (H,H) = 5.3 Hz, 1H), 2.80-2.58 (m, 4H), 1.84-1.78 (m, 1H), 1.74-1.62 (m, 2H), 1.59-1.48 (m, 2H), 1.36-1.28 (m, 1H), 0.96 (d, <sup>3</sup>J (H,H) = 6.3 Hz, 3H), 0.92 (d, <sup>3</sup>J (H,H) = 6.3 Hz, 3H), 0.86 (d, <sup>3</sup>J (H,H) = 6.1 Hz, 3H), 0.84 (d, <sup>3</sup>J (H,H) = 6.1 Hz, 3H); <sup>13</sup>C NMR (125 MHz, CDCl<sub>3</sub>): δ 175.0 (s), 172.9 (s), 172.1 (s), 171.0 (s), 170.9 (s), 170.3 (s), 156.2 (s), 144.3 (s), 144.2 (s), 143.8 (s), 143.7 (s), 141.5 (s), 141.4 (s), 128.8 (d), 128.7 (d), 128.1 (d), 128.1 (d), 127.9 (d), 127.3 (d), 127.2 (d), 125.1 (d), 125.0 (d), 120.2 (d), 71.1 (s), 71.0 (s), 67.4 (t), 53.7 (d), 52.7 (d), 51.1 (d), 51.0 (d), 47.0 (d), 40.1 (t), 40.0 (t), 38.9 (t), 36.8 (t), 24.9 (d), 24.8 (d), 23.3 (q), 23.0 (q), 21.6 (q), 21.5 (q); ESI-MS (MeOH/CH<sub>2</sub>Cl<sub>2</sub> 1:1): m/z 1200 (100, [M + Na]<sup>+</sup>).

**H-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub>.** Deprotection of Fmoc-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (1.31 g, 1.11 mmol) following the *general procedure B* and purification of the crude product by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 25:1) yielded pure H-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (984 mg, 93%) as colorless powder. TLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 10:1): R<sub>f</sub> 0.33; [α]<sup>20</sup><sub>D</sub> = -19.3 (c=1.01, CH<sub>2</sub>Cl<sub>2</sub>/MeOH 5:1); mp > 230 °C; IR: ν 3278 (m), 2957 (w), 1653 (s), 1629 (s), 1491 (s), 1445 (m), 1185 (m), 1036 (w), 751 (w), 697 (s); <sup>1</sup>H NMR (500 MHz, DMSO-d<sub>6</sub>/D<sub>2</sub>O 50:1): δ 7.28-7.15 (m, 30H), 4.51 (dd, <sup>3</sup>J (H,H) = 7.9 Hz, <sup>3</sup>J (H,H) = 6.3 Hz, 1H), 4.32 (dd, <sup>3</sup>J (H,H) = 10.9 Hz, <sup>3</sup>J (H,H) = 4.0 Hz, 1H), 4.16 (dd, <sup>3</sup>J (H,H) = 14.5 Hz, <sup>3</sup>J (H,H) = 8.8 Hz, 1H), 3.53 (dd, <sup>3</sup>J (H,H) = 9.8 Hz, <sup>3</sup>J (H,H) = 6.3 Hz, 1H), 2.74 (dd, <sup>2</sup>J (H,H) = 14.8 Hz, <sup>3</sup>J (H,H) = 6.3 Hz, 1H), 2.55 (dd, <sup>2</sup>J (H,H) = 14.8 Hz, <sup>3</sup>J (H,H) = 7.9 Hz, 1H), 2.48 (dd, <sup>2</sup>J (H,H) = 15.0 Hz, <sup>3</sup>J (H,H) = 6.3 Hz, 1H), 2.42 (dd, <sup>2</sup>J (H,H) = 15.0 Hz, <sup>3</sup>J (H,H) = 9.8 Hz, 1H), 1.63-1.40 (m, 6H), 0.87-0.80 (m, 12H); <sup>13</sup>C NMR (125 MHz, CD<sub>3</sub>OD): δ 174.1 (s), 173.9 (s), 172.1 (s), 170.5 (s), 170.2 (s), 169.2 (s), 145.0 (s), 144.7 (s), 128.6 (d), 128.5 (d), 127.5 (d), 127.4 (d), 126.3 (d), 126.3 (d), 69.4 (s), 69.3 (s), 52.0 (d), 50.9 (d), 50.9 (d), 50.0 (d), 41.4 (t), 41.1 (t), 40.7 (t), 37.3 (t), 24.1 (d), 24.0 (d), 23.2 (q), 23.1 (q), 21.7 (q), 21.4 (q); ESI-MS (MeOH/CH<sub>2</sub>Cl<sub>2</sub> 1:1): m/z 1935 (36, [2M + Na]<sup>+</sup>), 979 (100, [M + Na]<sup>+</sup>).

**Z-Leu-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub>.** Coupling of H-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (319 mg, 0.33 mmol) with Z-Leu-OH (127 mg, 0.48 mmol) following the *general procedure A* and purification of the crude product by column chromatography (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 25:1) and PTLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 20:1) yielded pure Z-Leu-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (314 mg, 79%) as colorless powder. TLC (CH<sub>2</sub>Cl<sub>2</sub>/MeOH 15:1): R<sub>f</sub> 0.26; HPLC (YMC-Pack SIL, 250 x 4.6 mm, CH<sub>2</sub>Cl<sub>2</sub>/MeOH 98:2, 1 ml/min, t<sub>R</sub> = 4.1 min); [α]<sup>20</sup><sub>D</sub> = -29.1 (c=0.129, CH<sub>2</sub>Cl<sub>2</sub>/MeOH 5:1); mp > 230 °C; IR: ν 3280 (m), 2957 (m), 1658 (s), 1636 (s), 1491 (s), 1447 (m), 1214 (m), 1037 (m), 695 (m); <sup>1</sup>H NMR (500 MHz, CD<sub>3</sub>OD): δ 7.25-7.09 (m, 35H), 5.01 (d, <sup>2</sup>J (H,H) = 12.2 Hz, 1H), 4.94 (d, <sup>2</sup>J (H,H) = 12.2 Hz, 1H), 4.44 (dd, <sup>3</sup>J (H,H) = 8.1

Hz,  $^3J$  (H,H) = 5.9 Hz, 1H), 4.42 (dd,  $^3J$  (H,H) = 7.2 Hz,  $^3J$  (H,H) = 5.4 Hz, 1H), 4.29 (dd,  $^3J$  (H,H) = 10.5 Hz,  $^3J$  (H,H) = 3.9 Hz, 1H), 4.11 (dd,  $^3J$  (H,H) = 9.7 Hz,  $^3J$  (H,H) = 5.2 Hz, 1H), 4.02 (dd,  $^3J$  (H,H) = 7.6 Hz,  $^3J$  (H,H) = 7.3 Hz, 1H), 2.86 (dd,  $^2J$  (H,H) = 15.2 Hz,  $^3J$  (H,H) = 7.2 Hz, 1H), 2.69 (dd,  $^2J$  (H,H) = 15.2 Hz,  $^3J$  (H,H) = 5.4 Hz, 1H), 2.60 (dd,  $^2J$  (H,H) = 15.7 Hz,  $^3J$  (H,H) = 5.9 Hz, 1H), 2.20 (dd,  $^2J$  (H,H) = 15.7 Hz,  $^3J$  (H,H) = 8.1 Hz, 1H), 1.68-1.43 (m, 9H), 0.93 (d,  $^3J$  (H,H) = 5.8 Hz, 6H), 0.89 (d,  $^3J$  (H,H) = 4.6 Hz, 6H), 0.82 (d,  $^3J$  (H,H) = 6.6 Hz, 6H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  176.9 (s), 174.8 (s), 174.2 (s), 172.9 (s), 172.5 (s), 170.9 (s), 170.4 (s), 157.9 (s), 145.1 (s), 145.0 (s), 136.9 (s), 129.4 (d), 129.3 (d), 129.0 (d), 128.6 (d), 128.4 (d), 128.3 (d), 128.2 (d), 127.6 (d), 127.5 (d), 71.3 (s), 71.1 (s), 67.5 (t), 54.7 (d), 53.9 (d), 52.6 (d), 51.7 (d), 51.4 (d), 41.3 (t), 40.5 (t), 40.3 (t), 38.0 (t), 38.0 (t), 25.4 (d), 25.3 (d), 25.2 (d), 23.5 (q), 23.3 (q), 23.2 (q), 21.8 (q), 21.6 (q), 21.5 (q); ESI-MS ( $\text{MeOH}/\text{CH}_2\text{Cl}_2$  1:1):  $m/z$  2430 (75,  $[2\text{M} + \text{Na}]^+$ ), 1226 (100,  $[\text{M} + \text{Na}]^+$ ).

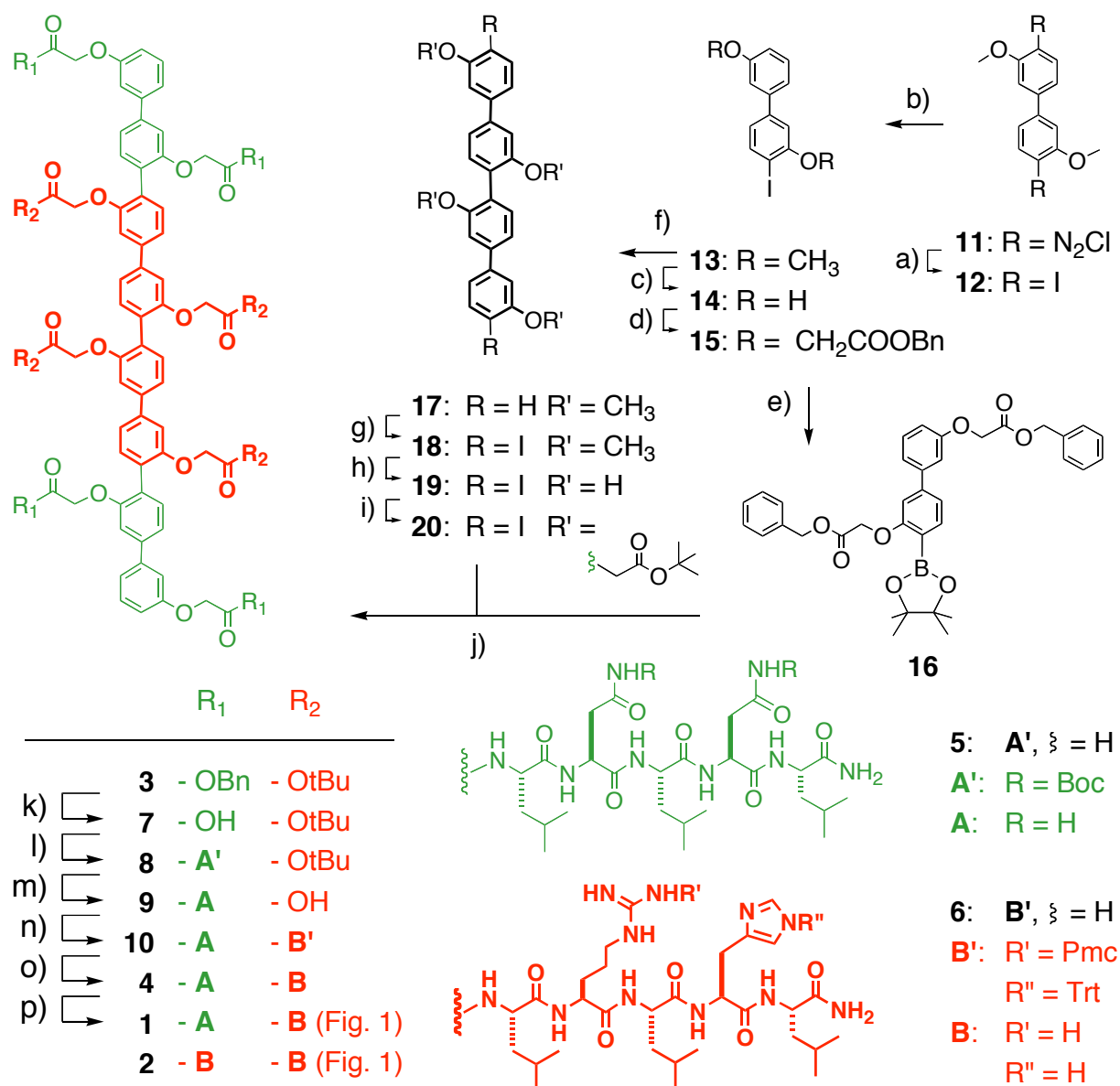
**H-Leu-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub> 5.** Deprotection of Z-Leu-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub> (82.8 mg, 0.069 mmol) following the *general procedure C* and purification of the crude product by column chromatography ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  12:1 then 7:1) and PTLC ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  10:1) yielded pure **5** (984 mg, 93%) as colorless powder. TLC ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  10:1):  $R_f$  0.25; HPLC (YMC-Pack SIL, 250 x 4.6 mm,  $\text{CH}_2\text{Cl}_2$ (0.1% TEA)/ $\text{MeOH}$  97:3, 1 ml/min,  $t_R$  = 3.4 min);  $[\alpha]_D^{20}$  = -17.4 (c=0.66,  $\text{CH}_2\text{Cl}_2/\text{MeOH}$  5:1); mp > 230 °C; IR:  $\nu$  3286 (m), 2956 (m), 1634 (s), 1490 (s), 1446 (m), 1186 (m), 1036 (w), 697 (s);  $^1\text{H}$  NMR (500 MHz,  $\text{DMSO}-d_6/\text{D}_2\text{O}$  50:1):  $\delta$  7.33-7.27 (m, 12H), 7.25-7.17 (m, 18H), 4.61 (dd,  $^3J$  (H,H) = 8.9 Hz,  $^3J$  (H,H) = 4.8 Hz, 1H), 4.52 (dd,  $^3J$  (H,H) = 7.3 Hz,  $^3J$  (H,H) = 7.3 Hz, 1H), 4.30 (dd,  $^3J$  (H,H) = 9.1 Hz,  $^3J$  (H,H) = 4.9 Hz, 1H), 4.19 (dd,  $^3J$  (H,H) = 9.1 Hz,  $^3J$  (H,H) = 6.0 Hz, 1H), 3.25 (dd,  $^3J$  (H,H) = 9.4 Hz,  $^3J$  (H,H) = 5.0 Hz, 1H), 2.82-2.72 (m, 2H), 2.65 (dd,  $^2J$  (H,H) = 14.9 Hz,  $^3J$  (H,H) = 4.8 Hz, 1H), 2.48 (dd,  $^2J$  (H,H) = 15.2 Hz,  $^3J$  (H,H) = 7.3 Hz, 1H), 1.82-1.72 (m, 1H), 1.66-1.54 (m, 2H), 1.52-1.40 (m, 5H), 1.30-1.22 (m, 1H), 0.94-0.82 (m, 18H);  $^{13}\text{C}$  NMR (125 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  175.6 (s), 175.3 (s), 172.5 (s), 171.0 (s), 170.9 (s), 169.4 (s), 169.1 (s), 143.4 (s), 127.9 (d), 126.9 (d), 126.0 (d), 69.8 (s), 69.7 (s), 52.0 (d), 51.6 (d), 51.1 (d), 50.0 (d), 49.5 (d), 39.2 (t), 39.2 (t), 39.1 (t), 39.0 (t), 37.0 (t), 36.5 (t), 23.8 (d), 23.7 (d), 23.6 (d), 22.0 (q), 21.8 (q), 21.6 (q), 20.5 (q), 20.0 (q), 20.0 (q); ESI-MS ( $\text{MeOH}/\text{CH}_2\text{Cl}_2$  1:1):  $m/z$  1091 (40,  $[\text{M} + \text{Na}]^+$ ), 1069 (100,  $[\text{M} + \text{H}]^+$ ).

**H-Leu-Arg(Pmc)-Leu-His(Trt)-Leu-NH<sub>2</sub> 6.** This compound was prepared in 8 steps following previously reported procedures.<sup>S2</sup>

**1<sup>3</sup>,2<sup>3</sup>,3<sup>2</sup>,4<sup>3</sup>,5<sup>2</sup>,6<sup>3</sup>,7<sup>2</sup>,8<sup>3</sup>-Octakis(Gla-Leu-Arg-Leu-His-Leu-NH<sub>2</sub>)-p-octiphenyl.** The monomer of pore **2** was prepared in 19 steps following previously reported procedures.<sup>S2</sup>

**1<sup>3</sup>,2<sup>3</sup>,7<sup>2</sup>,8<sup>3</sup>-Tetrakis(Gla-OBn)-3<sup>2</sup>,4<sup>3</sup>,5<sup>2</sup>,6<sup>3</sup>-tetrakis(Gla-OtBu)-p-octiphenyl 3.** This compound was prepared in 10 steps following previously reported procedures.<sup>S3</sup>

**1<sup>3</sup>,2<sup>3</sup>,7<sup>2</sup>,8<sup>3</sup>-Tetrakis(Gla-OH)-3<sup>2</sup>,4<sup>3</sup>,5<sup>2</sup>,6<sup>3</sup>-tetrakis(Gla-OtBu)-p-octiphenyl 7.** Pd(OH)<sub>2</sub>/C (1 mg) was added to a solution of **3** (13 mg, 7.3  $\mu\text{mol}$ ) in a mixture of MeOH (5 ml) and THF (5 ml), and the suspension was degassed before being set under a H<sub>2</sub>-atmosphere. After stirring for 2 h, the reaction mixture was filtered through celite and basic alumina. Concentration of the filtrate *in vacuo* yielded pure **7** (10.5 mg, 100%) as a colorless powder.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3/\text{CD}_3\text{OD}$  1:1):  $\delta$  7.51-7.43 (m, 6H), 7.39-7.15 (m, 18H), 6.94 (d,  $^3J$  (H,H) = 7.6 Hz, 2H), 4.67 (s, 4H), 4.65 (s, 4H), 4.62 (s, 4H), 4.61 (s, 4H), 1.48 (s, 36H).



**Scheme S1.** (a-j) see ref. S3; (k) H<sub>2</sub>, Pd(OH)<sub>2</sub>/C, MeOH/THF, 2 h, rt, quant; (l) **5**, HATU, TEA, DMF, 3 h, rt, 57%; (m) TFA, 3 h, rt, quant; (n) **6**, HATU, TEA, DMF, 3 h, rt, 65%; (o) TFA, 3 h, rt, quant; (p) self-assembly in lipid bilayers.

**1<sup>3</sup>,2<sup>3</sup>,7<sup>2</sup>,8<sup>3</sup>-Tetrakis(Gla-Leu-Asn(Trt)-Leu-Asn(Trt)-Leu-NH<sub>2</sub>)-3<sup>2</sup>,4<sup>3</sup>,5<sup>2</sup>,6<sup>3</sup>-tetrakis(Gla-OtBu)-p-octiphenyl **8**.** A solution of **5** (27 mg, 25.2 μmol) in DMF (610 μl) was added to **7** (6 mg, 4.2 μmol), HATU (13 mg, 33.6 μmol) and TEA (14 μl, 100 μmol). After stirring for 3 h at rt, the solvent was evaporated and the crude product dried *in vacuo*. Purification of the crude product by PTLC (first with CHCl<sub>3</sub>/MeOH 4:1, R<sub>f</sub> = 0.9, then with CHCl<sub>3</sub>/ MeOH 20:1, R<sub>f</sub> = 0.1) yielded HPLC-pure (YMC-Pack SIL, 250 x 4.6 mm, CHCl<sub>3</sub>/ MeOH 93:7, 1 ml/min, t<sub>R</sub> = 4.77 min) **8** (13.5 mg, 57%) as a colorless solid. <sup>1</sup>H NMR (300 MHz, CDCl<sub>3</sub>/CD<sub>3</sub>OD 1:1): δ 8.33-8.29 (m, ≤ 28 NH (slow exchange)), 7.93-7.73 (m, 8H), 7.49-7.45 (m, 4H), 7.42-7.39 (m, 10H), 7.26-7.09 (m, 122H), 6.89 (d, <sup>3</sup>J (H,H) = 8.3 Hz, 2H), 4.58-4.36 (m, 26H), 4.35-4.27 (m, 4H), 4.24-4.15 (m, 6H), 2.88-2.60 (m, 12H), 2.32-2.21 (m, 4H), 1.75-1.48 (m,

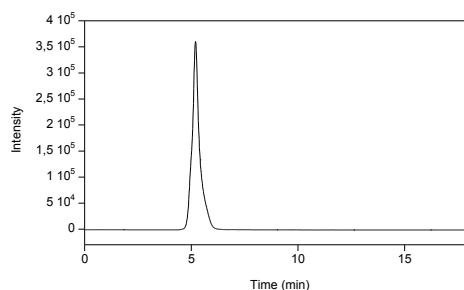
30H), 1.46 (s, 18H), 1.44 (s, 18H), 1.40-1.28 (m, 6H), 0.99-0.80 (m, 60H), 0.63 (d,  $^3J(\text{H,H}) = 5.52$  Hz, 6H), 0.59 (d,  $^3J(\text{H,H}) = 6.49$  Hz, 6H); ESI-MS ( $\text{CH}_2\text{Cl}_2/\text{MeOH}$  9:1):  $m/z$  1902 (100,  $[\text{M}+3\text{Na}^+]^{3+}$ ), 1432 (57,  $[\text{M}+4\text{Na}^+]^{4+}$ ).

**1<sup>3</sup>,2<sup>3</sup>,7<sup>2</sup>,8<sup>3</sup>-Tetrakis(Gla-Leu-Asn-Leu-Asn-Leu-NH<sub>2</sub>)-3<sup>2</sup>,4<sup>3</sup>,5<sup>2</sup>,6<sup>3</sup>-tetrakis(Gla-OH)-*p*-octiphenyl 9.** A solution of **8** (12.5 mg, 2.2  $\mu\text{mol}$ ) in TFA (2 ml) was stirred for 1.5 h at rt. After evaporation, impurities were removed by solid-liquid extraction with hexane (5 x 2 ml) and dried. Then, TFA (2 ml) was added and the solution was stirred for 1.5 h at rt. Following the addition of  $\text{CH}_2\text{Cl}_2$  (1 ml) and evaporation of the solvent, impurities were removed by solid-liquid extraction with hexane (1 ml, 4x), toluene (1 ml, 2x) and  $\text{CH}_2\text{Cl}_2$  (1 ml, 3x) to give HPLC-pure **9** (7.7 mg, 100%).  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3/\text{CD}_3\text{OD}$  1:1):  $\delta$  7.52-7.23 (m, 22H), 7.19-7.13 (m, 4H), 6.88 (d,  $^3J(\text{H,H}) = 7.81$  Hz, 2H), 5.06-4.42 (m, 36H), 3.12-2.89 (m, 16H), 1.78-1.23 (m, 36H), 0.92-0.62 (m, 72H).

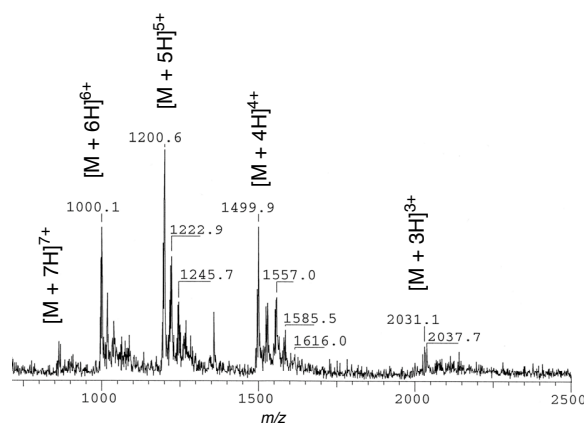
**1<sup>3</sup>,2<sup>3</sup>,7<sup>2</sup>,8<sup>3</sup>-Tetrakis(Gla-Leu-Asn-Leu-Asn-Leu-NH<sub>2</sub>)-3<sup>2</sup>,4<sup>3</sup>,5<sup>2</sup>,6<sup>3</sup>-tetrakis(Gla-Leu-Arg(Pmc)-Leu-His(Trt)-Leu-NH<sub>2</sub>)-*p*-octiphenyl 10.** A solution of **6** (15.4 mg, 13.3  $\mu\text{mol}$ ) in DMF (500  $\mu\text{l}$ ) was added to **9** (7.7 mg, 2.2  $\mu\text{mol}$ ), HATU (6.7 mg, 17.7  $\mu\text{mol}$ ) and TEA (7.4  $\mu\text{l}$ , 53.2  $\mu\text{mol}$ ). After stirring for 3 h at rt, the solvent was evaporated and the product dried *in vacuo*. Impurities were removed by solid-liquid extraction with EtOAc (1 ml, 3x). Purification of the crude by column chromatography (LH-20 sephadex column, DMF) and RP-HPLC (YMC-Pack ODS-A, 250 x 410 mm, MeOH, 2 ml/min,  $t_R = 4.18$  min) gave HPLC-pure **10** (11.5 mg, 65%) as a colorless solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CDCl}_3/\text{CD}_3\text{OD}/\text{DMF}$  20:20:1):  $\delta$  7.58-6.97 (m, 94H), 5.06-4.17 (m, 56H), 3.24-2.65 (m, 32H), 2.64-2.44 (m, 32H), 2.07-1.97 (m, 12H), 1.82-1.24 (m, 96 H), 1.28-1.22 (m, 24H), 1.01-0.57 (m, 144H).

**1<sup>3</sup>,2<sup>3</sup>,7<sup>2</sup>,8<sup>3</sup>-Tetrakis(Gla-Leu-Asn-Leu-Asn-Leu-NH<sub>2</sub>)-3<sup>2</sup>,4<sup>3</sup>,5<sup>2</sup>,6<sup>3</sup>-tetrakis(Gla-Leu-Arg-Leu-His-Leu-NH<sub>2</sub>)-*p*-octiphenyl 4.** A solution of **10** (2.0 mg, 0.25  $\mu\text{mol}$ ) in TFA (2 ml) was stirred for 1.5 h at rt. After addition of  $\text{CH}_2\text{Cl}_2$  (1 ml), evaporation of the solvents and solid-liquid extraction of impurities with hexane (2 ml, 5x), TFA (2 ml) was added and the solution was stirred for 1.5 h at rt, diluted with  $\text{CH}_2\text{Cl}_2$  (1 ml) and the solvents were evaporated. Impurities were removed by solid-liquid extraction with hexane (1 ml, 4x), toluene (1 ml, 2x) and  $\text{CH}_2\text{Cl}_2$  (1 ml, 3x). The crude product was purified by RP-HPLC (YMC-Pack ODS-A, 250 x 10 mm, MeOH/  $\text{H}_2\text{O}$ / TFA 92:8:1, 2 ml/min,  $t_R = 5.20$ ) to give HPLC-pure (Fig. S1) **4** (1.5 mg, 100%) as a colorless solid.  $^1\text{H}$  NMR (300 MHz,  $\text{CD}_3\text{OD}$ ):  $\delta$  7.64-7.20 (m, 36H), 7.09-7.04 (m, 2H), 4.80-4.20 (m, 56H), 3.26-2.55 (m, 32H), 1.82-1.27 (m, 88H), 1.04-0.64 (m, 144H); ESI-MS ( $\text{MeOH}/\text{CH}_3\text{CN}/\text{AcOH}$  74:24:2):  $m/z$  2038 (11,  $[\text{M}+3\text{H}+\text{TFA}]^{3+}$ ), 2033 (8,  $[\text{M}+3\text{H}+\text{H}_3\text{PO}_4]^{3+}$ ), 3032 (11,  $[\text{M}+3\text{H}+\text{TFA}-\text{H}_2\text{O}/\text{NH}_3]^{3+}$ ), 1586 (21,  $[\text{M}+4\text{H}+3\text{TFA}]^{4+}$ ), 1581 (17,  $[\text{M}+4\text{H}+2\text{TFA}+\text{H}_3\text{PO}_4]^{4+}$ ), 1580 (17,  $[\text{M}+4\text{H}+3\text{TFA}-\text{H}_2\text{O}/\text{NH}_3]^{4+}$ ), 1577 (12,  $[\text{M}+4\text{H}+\text{TFA}+2\text{H}_3\text{PO}_4]^{4+}$ ), 1577 (12,  $[\text{M}+4\text{H}+2\text{TFA}+\text{H}_3\text{PO}_4-\text{H}_2\text{O}/\text{NH}_3]^{4+}$ ), 1557 (38,  $[\text{M}+4\text{H}+2\text{TFA}]^{4+}$ ), 1553 (33,  $[\text{M}+4\text{H}+\text{TFA}+\text{H}_3\text{PO}_4]^{4+}$ ), 1552 (33,  $[\text{M}+4\text{H}+2\text{TFA}-\text{H}_2\text{O}/\text{NH}_3]^{4+}$ ), 1548 (12,  $[\text{M}+4\text{H}+\text{TFA}+\text{H}_3\text{PO}_4-\text{H}_2\text{O}/\text{NH}_3]^{4+}$ ), 1529 (30,  $[\text{M}+4\text{H}+\text{TFA}]^{4+}$ ), 1525 (16,  $[\text{M}+4\text{H}+\text{H}_3\text{PO}_4]^{4+}$ ), 1524 (39,  $[\text{M}+4\text{H}+\text{TFA}-\text{H}_2\text{O}/\text{NH}_3]^{4+}$ ), 1520 (13,  $[\text{M}+4\text{H}+\text{H}_3\text{PO}_4-\text{H}_2\text{O}/\text{NH}_3]^{4+}$ ), 1500 (75,  $[\text{M}+4\text{H}]^{4+}$ ), 1496 (34,  $[\text{M}+4\text{H}-\text{H}_2\text{O}/\text{NH}_3]^{4+}$ ), 1491 (15,  $[\text{M}+4\text{H}-2\text{H}_2\text{O}/\text{NH}_3]^{4+}$ ), 1268 (14,  $[\text{M}+5\text{H}+3\text{TFA}]^{5+}$ ), 1265 (17,  $[\text{M}+5\text{H}+2\text{TFA}+\text{H}_3\text{PO}_4]^{5+}$ ), 1265 (17,  $[\text{M}+5\text{H}+3\text{TFA}-\text{H}_2\text{O}/\text{NH}_3]^{5+}$ ), 1262 (11,  $[\text{M}+5\text{H}+\text{TFA}+2\text{H}_3\text{PO}_4]^{5+}$ ), 1261 (20,  $[\text{M}+5\text{H}+2\text{TFA}+\text{H}_3\text{PO}_4-\text{H}_2\text{O}/\text{NH}_3]^{5+}$ ), 1246 (26,  $[\text{M}+5\text{H}+2\text{TFA}]^{5+}$ ), 1242 (21,  $[\text{M}+5\text{H}+\text{TFA}+\text{H}_3\text{PO}_4]^{5+}$ ), 1242 (21,  $[\text{M}+5\text{H}+2\text{TFA}-\text{H}_2\text{O}/\text{NH}_3]^{5+}$ ), 1239 (13,

[M+5H+TFA+H<sub>3</sub>PO<sub>4</sub>-H<sub>2</sub>O/NH<sub>3</sub>]<sup>5+</sup>, 1222 (40, [M+5H+TFA]<sup>5+</sup>), 1220 (33, [M+5H+H<sub>3</sub>PO<sub>4</sub>]<sup>5+</sup>), 1219 (30, [M+5H+TFA-H<sub>2</sub>O/NH<sub>3</sub>]<sup>5+</sup>), 1216 (25, [M+5H+H<sub>3</sub>PO<sub>4</sub>-H<sub>2</sub>O/NH<sub>3</sub>]<sup>5+</sup>), 1200 (100, [M+5H]<sup>5+</sup>), 1197 (44, [M+5H-H<sub>2</sub>O/NH<sub>3</sub>]<sup>5+</sup>), 1193 (16, [M+5H-2H<sub>2</sub>O/NH<sub>3</sub>]<sup>5+</sup>), 1038 (13, [M+6H+2TFA]<sup>6+</sup>), 1019 (13, [M+6H+TFA]<sup>6+</sup>), 1017 (14, [M+6H+H<sub>3</sub>PO<sub>4</sub>]<sup>6+</sup>), 1016 (21, [M+6H+TFA-H<sub>2</sub>O/NH<sub>3</sub>]<sup>6+</sup>), 1014 (14, [M+6H+H<sub>3</sub>PO<sub>4</sub>-H<sub>2</sub>O/NH<sub>3</sub>]<sup>6+</sup>), 1001 (88, [M+6H]<sup>6+</sup>), 998 (36, [M+6H-H<sub>2</sub>O/NH<sub>3</sub>]<sup>6+</sup>), 995 (13, [M+6H-2H<sub>2</sub>O/NH<sub>3</sub>]<sup>6+</sup>) (Fig. S2).



**Figure S1.** HPLC of target molecule **4** (detection at 254 nm).



**Figure S2.** ESI-MS of target molecule **4** (TFA salt) under denaturing acidic conditions (MeOH/CH<sub>3</sub>CN/AcOH 74:24:2).

*Fig. S2, comments:* For counteranion scavenging, see ref (S4).

**EYPC-LUVs**  $\Delta$ ANTS/DPX. Solutions of EYPC (25 mg) in CHCl<sub>3</sub>/MeOH 1/1 (2 ml) were dried using rotary evaporator and then under vacuum (>2 h) to form thin films. The resulting films were hydrated with 1 ml buffer (12.5 mM ANTS, 45.0 mM DPX, 5 mM TES, 20 mM KCl, pH 7.0) for more than 30 min and subjected to freeze-thaw cycles (5x) and extrusions (15x, Mini-Extruder with a polycarbonate membrane, pore size 100 nm). Extravesicular dyes were removed by gel filtration (Sephadex G-50; 5 mM TES, 100 mM KCl, pH 7.0; external cation exchange using 5 mM TES, 100 mM NaCl, pH 7.0 was done for polarized LUVs; see below). The LUV fractions were combined and diluted to 6 ml with the same buffers. Lipid concentrations were estimated from amount of entrapped dye. The estimated values were consistent with earlier results from phosphate analysis.<sup>S5</sup> The final stock solutions had the following characteristics: ~1.3 mM EYPC; 12.5 mM ANTS, 45.0 mM DPX, 5 mM TES, 20 mM KCl, pH 7.0 inside, 5 mM TES, 100 mM KCl or NaCl, pH 7.0 outside.

**Vesicle Polarization.**<sup>S6</sup> 50  $\mu$ l of LUV-stock solution was diluted with a mixture of  $k$   $\mu$ l of 100 mM KCl, 10 mM MES, pH 4.5-7.0, and 1950 -  $k$   $\mu$ l of 100 mM NaCl, 10 mM MES, pH 4.5-7.0, containing safranin O (60 nM). Membrane potential  $V$  was calculated from the Nernst equation [S1]

$$V \text{ (mV)} = 59 \times \log ([K^+_{\text{out}}] / [K^+_{\text{in}}]) \quad [\text{S1}],$$

$$= -153.5 + 59 \times \log k \quad [\text{S1}']$$

assuming external  $[K^+_{\text{out}}] \approx 0$  in the LUV-stock solution. For calibration, the emission of safranin O was monitored at  $\lambda_{\text{em}}$  581 nm ( $\lambda_{\text{ex}}$  522 nm) as a function of time during addition of valinomycin (20  $\mu$ l of 60  $\mu$ M, final 600 nM), incubation for  $\sim$ 100 sec (until emission of polarized vesicles was constant) and successive addition of external  $K^+$  buffer. Linear correlation was found between the emission intensities and the calculated Nernst potential from  $-80$  to  $-190$  mV.<sup>S6,S7</sup> Extrapolation to  $k = 0$   $\mu$ l gave  $V = -211$  mV for a Nernst potential of  $-\infty$  mV, indicative for residual external  $K^+$  in the LUV-stock solution or “saturation” (S6). The magnitude of vesicle polarization was determined in each experiment by comparison of the measured Safranin-O emission intensity after the addition of valinomycin with this calibration curve.

**ANTS/DPX-assay. Unpolarized LUVs.** EYPC-LUVs $\supset$ ANTS/DPX (50  $\mu$ l) were added to gently stirred, thermostated buffer (1.95 ml; 100 mM KCl, 10 mM MES, pH 4.5-7.0, 25  $^\circ$ C). Changes in fluorescence emission of ANTS ( $F_t$ ,  $\lambda_{\text{em}} = 510$  nm,  $\lambda_{\text{ex}} = 353$  nm; similar to top traces in Fig. 2A) was monitored (FluoroMax-3, Jobin Yvon) as a function of time during addition of blocker (at time 0, ADP, ATP, phytate, varied concentrations), barrel **1** (usually 250 nM; for concentration dependence 0-250 nM: see Fig. S3B) or **2** (75 nM) at 2 min and 40  $\mu$ l of 1.2 % Triton X-100 at 9.5 min. Precise control of temperature and continuous stirring during fluorescence kinetics measurements were crucial for reproducible results. *Polarized LUVs.* EYPC-LUVs $\supset$ ANTS/DPX (50  $\mu$ l, external salts exchanged to  $\text{Na}^+$ ) were added to gently stirred, thermostated buffer ( $k$   $\mu$ l of 100 mM KCl, 5 mM MES, 1950- $k$   $\mu$ l of 100 mM NaCl, 5 mM MES, pH 4.5-7.0, 25  $^\circ$ C). Changes in fluorescence emission of ANTS ( $\lambda_{\text{em}} = 510$  nm,  $\lambda_{\text{ex}} = 353$  nm; top traces in Fig. 2A) and safranin O ( $\lambda_{\text{em}}$  581 nm,  $\lambda_{\text{ex}}$  522 nm, bottom traces in Fig. 2A) were monitored simultaneously in two different channels as a function of time during addition of safranin O (60 nM final), blocker (ADP, ATP, phytate, varied concentrations), valinomycin (0.6  $\mu$ M; Fig. 2A: (c) = 1 min), barrel **1** (250 nM) or **2** (75 nM) at time (d) (Fig. 2A: (d) = 5 min), and 40  $\mu$ l melittin (1.3 mg / ml  $\text{H}_2\text{O}$ ) at time (e) (Fig. 2A: (e) = 9.5 min).

**Data analysis.** Fluorescence time courses were normalized to fractional emission intensity  $I^n$  using equation [S2]

$$I^n = (F_t - F_0) / (F_\infty - F_0) \quad [\text{S2}],$$

where  $F_0 = F_t$  at pore addition,  $F_\infty = F_t$  at saturation after lysis. If appropriate, fractional pore activity  $Y$  was calculated using equation [S3]

$$Y = I^n / I^n_{\text{MAX}} \quad [\text{S3}],$$



where  $I_{MAX}^n$  is  $I^n$  at saturation obtained under the conditions giving the highest activity, and used instead of the fractional emission intensity  $I^n$ . Effective monomer concentrations  $EC_{50}$  and thermodynamic pore stability and/or<sup>S8</sup> stoichiometry  $n$  were determined from a fit of the dependence of pore activity on monomer concentration  $c_M$  to the Hill equation [S4]

$$I^n = I_{MAX}^n + (I_0^n - I_{MAX}^n) / \{1 + c_M / EC_{50}\}^n \quad [S4],$$

where  $I_0^n$  is  $I^n$  without pore,  $I_{MAX}^n$  is  $I^n$  four minutes after addition of excess pore and  $n$  is the Hill coefficient (pH,  $V$ ,  $T$ , = constant). Gating charges  $z_g$  were determined from the fit of the dependence of pore activity on vesicle polarization  $V$  (at constant pore concentration = 250 nM and constant pH = 5.5) to equation [S5]

$$Y \propto \exp(z_g e V / k T) \quad [S5]$$

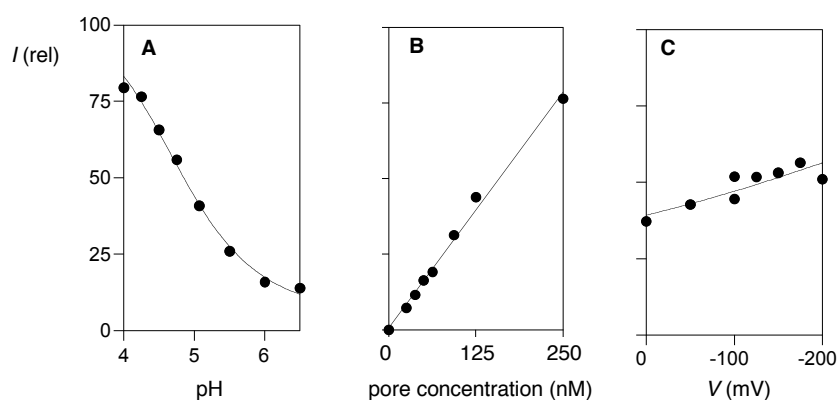
where  $e$  is the elementary charge,  $k$  the Boltzmann constant, and  $T$  the absolute temperature (pH,  $T$ ,  $c_M$  = constant).<sup>S6</sup> Apparent dissociation constants  $K_D$  (more accurately: inhibitory concentrations  $IC_{50}$ ) were determined from the dependence of fractional pore activities  $Y$  at 400 sec on the blocker concentration and fitted to the Hill equation [S6]

$$Y = Y_\infty + (Y_0 - Y_\infty) / \{1 + c_{BLOCKER} / K_D\}^n \quad [S6],$$

where  $Y_0$  is  $Y$  without ligand,  $Y_\infty$  is  $Y$  with excess ligand and  $n$  is the Hill coefficient (pH,  $V$ ,  $T$ ,  $c_M$  = constant). The Woodhull distance  $l_w$  from pore entrance to active site  $OR$  the effective charge  $z_{eff}$  of the blocker were determined by the fit of the dependence of the apparent dissociation constant  $K_D$  on the vesicle polarization  $V$  to the Woodhull equation [S7]

$$pK_D = pK_D(0 \text{ mV}) - (z_{eff} l F / l_w 2.303 R T) V \quad [S7],$$

where  $l$  is the length of the pore (34 Å assumed),  $F$  the Faraday constant,  $R$  the gas constant and  $T$  the absolute temperature.<sup>S9</sup>



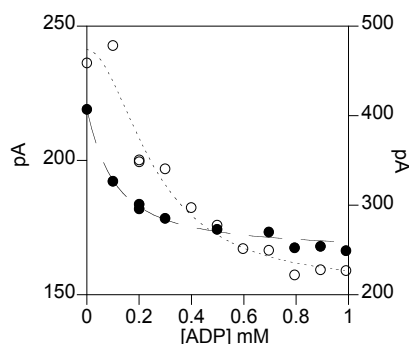
**Figure S3.** Dependence of the fractional activity of pore 1 on pH (A, 250 nM tetramer,  $V = 0$  mV) on concentration (B, curve fit to eqn [S4], pH = 4.25,  $V = 0$  mV), and on vesicle polarization (C, curve fit to eqn [S5], 250 nM tetramer, pH = 5.5).

*Fig. S3, comments:* (A) for pH profile of pore **2**, see (S10). (B) Based on extensive precedence,<sup>S8,S10</sup> pore concentration was approximated as monomer concentration  $c_M/4$ . Linear dependence indicated that pore **1** is formed by monomeric rods (unlikely) or by exergonic self-assembly (likely).<sup>S8,S10</sup> Absence of early saturation indicated that pore **1** has reduced thermodynamic stability compared to pore **2**.<sup>S8,S10</sup> (C) Curve fit to eqn [S5] resulted in the gating charge  $z_g$  0.047. This value indicated voltage independence of the pore **1**. For comparison of results on voltage gating from polarized vesicles with IV profiles from planar bilayer conductance experiments, see (S6).

**Planar Bilayer Conductance.** BLMs were formed by painting a solution of EYPC in *n*-decane (42 mg/ml) on an aperture ( $d = 150 \mu\text{m}$ , pretreated with the same solution) in a delrin cuvette separating two chambers containing 1 ml each of buffer (5mM MES 1.05 M KCl, pH 4.65) and agar bridge connection (2 M KCl) to Ag/AgCl electrodes (Warner Instrument Corp. Hamden, CT). Currents were recorded at different holding potentials (*trans* at ground) in a house-made Faraday cage, amplified (BC-525c, Warner Instrument Corp.), low-pass filtered with a 8-pole Bessel filter at 1 kHz (LPF-8, Warner Instrument Corp.), A-D converted (DigiData 1200, Axon Instruments, Union City, CA), and sampled at 10 kHz by computer (pClamp 8.0, Axon Instruments). Pore **1** (250 nM) was added to the *cis* chamber. All the conductance measurements were performed at room temperature ( $22 \pm 1 \text{ }^\circ\text{C}$ ). Conductance  $g$  was estimated from the histogram, multiplied by Sansom's correction factor  $(5.61)^{S11}$  and applied to Hille's equation<sup>S12</sup>

$$1 / g = (l + \pi d / 4) \times (4\rho / \pi d^2) \quad [\text{S8}]$$

where  $l$  is the ion channel length (34 Å) and  $\rho$  the resistivity of the recording solution ( $\rho = 7.8 \Omega\cdot\text{cm}$ ), to give the calculated channel diameter  $d$ , 0.7 nm (for pore **2**, see (S5)). To examine the effect of ADP to conductance of the pores, BLMs were prepared as described above in a buffer (10 mM MES, 0.1 M KCl, pH 4.5). Currents through multiple channels were measured at different holding potential in the presence of increasing amount of ADP in the *trans* chamber. The average currents over 100 ms ( $Y$ ) were fitted to the Hill equation [S6] (Figure S4) to give apparent  $K_D$ s. Thus obtained  $K_D$ s were applied to the Woodhull equation [S7] (Fig. 3B). Data points for pore **1** in Fig. 3B were the averages of two independent series of experiments  $\pm$  errors.



**Figure S4.** Representative multichannel dose response curves at 60 mV (open circles) and 100 mV (filled circles).

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