Supporting Information

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Designing Facial Amphiphiles for the Stabilization of Integral Membrane Proteins

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General procedure. NMR spectra were recorded on Bruker DRX-500, AMX-500 or AMX-300 instruments. IR spectra were recorded on a Perkin-Elmer 1600 series FT-IR spectrometer. High-resolution mass spectra (HRMS) were recorded on a VG ZAB-ZSE mass spectrometer using MALDI (matrix-assisted laser-desorption/ionization) or ESI (electrospray ionization).

3 α ,7 α ,12 α ,24-Tetrahydroxycholane (3):^[1] To a suspension of LiAlH₄ (3.85 g, 100 mmol) in dry THF (100 mL) at 0°C was added dropwise a solution of cholic acid (15.03 g, 35 mmol) in dry THF (200 mL) with vigorous stirring under nitrogen atmosphere. The reaction mixture was then heated to reflux with stirring for overnight. Upon completion, the reaction was carefully quenched with saturated aqueous NH₄Cl solution at RT. Then the mixture was acidified with 1N HCl to pH 1~2. The precipitate was collected via filtration and washed with water and acetone to give the product **2** (12.1 g, 88%) as a white solid.

7 α ,12 α -Dihydroxycholane (2): To a solution of tetrahydroxycholane **3** (17.4 g, 44.2 mmol) and triethylamine (11.8 g, 116.6 mmol) in dry THF (300 mL) was added dropwise a solution of methanesulfonyl chloride (11.1 g, 97.2 mmol) in dry THF (100 mL) at 0°C. Then the reaction mixture was slowly warmed up to RT. After 30 minutes, the reaction was quenched with saturated aqueous NH₄Cl solution. The organic solvents were removed under vacuum and the left aqueous solution was extracted with ethyl acetate. The combined organic portions were washed with brine and then dried over anhydrous Na₂SO₄. The filtered solution was concentrated under vacuum to give the product 7 α ,12 α -Dihydro-3 α ,24-dimethylsulfonate-cholane (25.6 g, 95%) which was directly dissolved in dry THF for the next step.

A solution of LiAlH₄ (6.0 g, 158 mmol) in dry THF (300 mL) was added dropwise to the above obtained THF solution of 7 α ,12 α -Dihydro-3 α ,24-dimethylsulfonate-cholane at 0°C. The reaction mixture was heated to reflux with stirring for overnight. Then the reaction was quenched with saturated aqueous NH₄Cl solution at RT. The organic solvents were evaporated under vacuum and the left aqueous solution was acidified with 1N HCl to pH 1. The white precipitate formed was collected via filtration, washed with water and acetone to give the crude product, and then crystallized in dichloromethane and methanol to afford the pure compound 7 α ,12 α -dihydrocholane (12.8 g, 80% over two steps). Mp: 182-184°C; [α]_D²⁵ = -333.3 (CHCl₃, *c* = 0.84); IR (film) ν_{max} = 3380, 2919, 2857, 2360, 2341, 1653, 1450, 1376, 1192, 1085, 1027, 984, 909 cm⁻¹; ¹H NMR (300 MHz, CDCl₃) δ = 4.01 (brs, 1H), 3.85 (brs, 1H), 2.20-1.80 (m, 5H), 1.78-1.00 (series of multiplet, 23 H), 0.98 (d, *J* = 6.3 Hz, 3H), 0.90-0.85 (m, 6H), 0.70 (s, 3H) ppm; ¹³C NMR (125 MHz, CDCl₃ and CD₃OD): δ 72.8, 68.1, 47.0, 45.9, 42.8, 41.2, 39.1, 37.8, 37.1, 35.2, 34.5, 29.9, 27.7, 27.2, 27.2, 26.0, 22.8, 22.7, 20.7, 18.9, 16.9, 13.8, 11.9 ppm; HR-MS: calcd for C₂₄H₄₂O₂Na⁺ [*M* + Na⁺]: 385.3077, found 385.3057.

7 α , 12 α -Di-(O- β -D-maltosyl)-cholane (1): A mixture of compound **2** (210 mg, 0.58 mmol), 1-thio-ethyl-hepta-*o*-benzoyl- β -D-maltose (2.1 g, 2.08 mmol) and 4 Å molecule sieves (800 mg) in dry CH₂Cl₂ (50 mL) was stirred at RT for 30 minutes. The reaction mixture was then cooled to -15 °C, to which was added crystallized *N*-iodosuccinimide (500 mg, 2.22 mmol) and silver trifluorosulfonate (100 mg, 0.39 mmol). The reaction mixture was slowly warmed up to RT with stirring. The reaction was monitored by TLC. Upon completion, the reaction was quenched with triethylamine. The mixture was filtered and the filtrate was concentrated under vacuum. The residue was submitted to column chromatography on silica gel to separate the crude mono- and di-glycosylation products. The mono-glycosylation products can be recovered for complete glycosylation. The combined di-glycosylation products were directly dissolved in methanol (30 mL), and to this solution was added sodium methoxide (300 mg) with stirring. The reaction was stirred at RT for overnight. The solvent was removed under vacuum and the residue was subject to column chromatography on silica gel to give compound **1** (305 mg, 52%) as a solid. Mp: 188-190°C; [α]_D²⁵ = 95.0 (MeOH, *c* = 1.00); IR (film) ν_{max} = 3351, 2920, 2361, 2340, 1646, 1375, 1146, 1073, 1019, 607 cm⁻¹; ¹H NMR (500 MHz, CD₃OD) δ = 5.18 (d, *J* = 4.0 Hz, 1H), 5.17 (d, *J* = 4.0 Hz, 1H), 5.56 (s, 1H), 4.37 (d, *J* = 8.0 Hz, 1H), 4.36 (d, *J* = 8.0 Hz, 1H), 3.93-3.73 (m, 9H), 3.71-3.59 (m, 8H), 3.53-3.43 (m, 4H), 3.39-3.23 (m, 8H), 2.31-1.09 (m, 25 H), 1.03 (d, *J* = 6.5 Hz, 3H), 0.92 (s, 3H), 0.86 (t, *J* = 7.0 Hz, 3H), 0.71 (s, 3H) ppm; ¹³C NMR (125 MHz, CD₃OD): 106.6, 102.8, 102.7, 100.9, 86.3, 81.7, 81.5, 78.1, 78.0, 76.6, 76.5, 75.2, 75.1, 75.0, 74.9, 74.7, 74.1, 71.5, 62.9, 62.8, 62.7, 62.6, 47.8, 47.0, 44.9, 43.3, 40.8, 39.6, 39.2, 37.7, 36.8, 31.2, 30.7, 29.3, 28.9, 28.2, 24.2, 24.1, 22.7, 20.3, 18.5, 15.0, 13.0 ppm; HR-MS: calcd for C₄₈H₈₂O₂₂Na⁺ [*M* + Na⁺]: 1033.5190, found 1033.5196.

Critical Micelle Concentration (CMC) Measurement.^[2] The CMC of compound **1** was determined by using fluorescence dye binding technique with 8-anilino-1-naphthalenesulfonic ammonium salt (ANS) as the probe molecule. The molecule ANS has a weak fluorescence in detergent concentrations below CMC, but becomes highly fluorescent when incorporated into the hydrophobic micellar environment. Solutions of ANS (10 μ M) and **1** at various concentrations in water were prepared. The emission fluorescence intensities at 465nm were recorded (λ_{ex} = 405 nm) on a DXT880 multiplate spectrofluorimeter (Beckman Coulter). The CMC is defined as the breakpoint in the fluorescence (Figure S1).

Figure S1. CMC determination of compound **1** by ANS binding ($\lambda_{\text{ex}} = 405 \text{ nm}$, $\lambda_{\text{em}} = 465 \text{ nm}$).

Measurement of Hydrodynamic Radius of Detergent Micelles. The micelles formed by DDM and compound **1** (same concentration at 0.03%) were measured on DynaPro/Titan instrument (Wyatt Technology Corporation) equipped with a plate reader and a laser operating at 658 nm.

MsbA Preparation. MsbA was prepared from *S. typhimurium* as described previously.^[3] The membranes were solubilized in 20 mM detergents (β -UDM and compound **1**, respectively) buffered with 20 mM Tris, 20 mM NaCl (pH 8.0). The supernatant after centrifugation at 200,000g for 45 min was subject to Ni-affinity column for purification. The protein extraction and purity was evaluated by SDS-PAGE. MsbA was further purified by anion-exchange chromatography and desalting column for the stability evaluation which was followed by ATPase activity measurement. MsbA (12-20 mg/mL) was stored in a pH 7.5 solution buffered with 20 mM Tris and 20 mM NaCl in the presence of 0.03% β -UDM and 0.02% compound **1**, respectively.

Preparation of Isolated Nucleotide Binding Domain. The NBD only construct (residues 330-582) was cloned into pET19b expression vector (Novagen), which contains a 23-residue fusion leader containing an N-terminal decahistidine tag to aid in purification, over-expressed in *E. coli* host BL21 (DE3) (Novagen) in 100 liter batch fermentors at 37°C using 2mM IPTG (Anatrace, Maumie, OH) as the inducer, and extracted by sonication at 4° C. Extracted NBD was purified with 10% glycerol by nickel-chelation followed by ion-exchange and gel-filtration chromatography to increase protein enrichment and purity. The protein was finally exchanged into 20mM Tris (pH 7.5), 100mM NaCl.

ATPase Activity. ATPase activity was measured using an ATP-regenerating system described by Vogel and Steinhart,^[4] and modified by Urbatsch et al.^[5] Briefly, 1-2 μg of detergent solubilized MsbA was added to 100 μL of Linked Enzyme (LE) buffer at 37 °C containing 10 mM ATP, 12 mM MgCl_2 , 6 mM phosphoenolpyruvate (PEP), 1 mM NADH, 10 units of lactate dehydrogenase (LDH), 10 units of pyruvate kinase (PK), and 50 mM Tris-HCl (pH 7.5). ATP hydrolysis was measured as the decrease in absorbance of NADH at 340nm using a DXT880 multiplate spectrofluorimeter (Beckman-Coulter). ATPase activity was calculated using the following equation: $\Delta\text{OD} \cdot \epsilon / ([\text{protein}] \cdot \text{time})$, where ΔOD is the change in optical density and ϵ is the extinction coefficient.

Detergent Binding Measurement Using Glycosidic Colormetric Assay. The detergent binding was determined according to the lit. procedure.^[6] Briefly, 50 μL detergent-containing samples were mixed with 200 μL of 5% phenol solution and 600 μL of concentrated sulfuric acid. The reaction produced orange-yellow color which had a maximal adsorption at 488 nm. A calibration curve of absorbance at 488nm as a function of detergent concentration was first established for β -UDM and compound **1**, respectively (Figure S2). The above purified MsbA fractions in β -UDM and compound **1** from sephadex gel column without involving concentration step were used to determine the total detergent concentration. The molar ratio of detergent binding was calculated using the following equation: $[(C_{\text{det, total}} - C_{\text{det, baseline}}) / [\text{MW}]_{\text{det}}] / [C_{\text{MsbA}} / 128,000]$.

Figure S2. (a) Spectra obtained from detergents reacted with phenol and sulphuric acid. (b) standard curves from UDM and compound **1** in colormetric assay.

Preparation of bacteriorhodopsin (BR). BR was isolated from purple membrane of *Halobacterium halobium* (strain S9) and purified in 1.2% β -octyl-glycoside (OG) as reported in literature.^[7] The concentrated BR stock (13.5 mg/mL) in 1.2% OG (25 mM potassium phosphate, pH 5.6) was diluted to 0.02% compound **1** in the same buffer. After several dilution-concentration cycles, the final OG

concentration was <1% of its CMC (0.53%). BR samples (0.2 mg/mL) were incubated in compound **1** (0.02%) and OG (1.2%) at RT in the dark. The UV-Vis absorption spectra of BR samples were recorded on a DXT880 multiplate spectrofluorimeter (Beckman Coulter).

References:

- [1] Li, H.; Wang, L. X. *Or.g Biomol. Chem.* **2003**, *1*, 3507-13.
- [2] De Vendittis, E.; Palumbo, G.; Parlato, G.; Bocchini, V. *Anal. Biochem.* **1981**, *115*, 278-86.
- [3] Reyes, C. L.; Chang, G. *Acta Crystallograph Sect. F Struct. Biol. Cryst. Commun.* **2005**, *61*, 655-8.
- [4] Vogel, G.; Steinhart, R. *Biochemistry* **1976**, *15*, 208-16.
- [5] Urbatsch, I. L.; Sankaran, B.; Weber, J.; Senior, A. E. *J. Biol. Chem.* **1995**, *270*, 19383-90.
- [6] A. Urbani, T. Warne, *Anal. Biochem.* **2005**, *336*, 117-124.
- [7] Landau, E. M.; Rosenbusch, J. P. *Proc. Natl. Acad. Sci. USA* **1996**, *93*, 14532-5.

Complete reference [2f] in the main text:

- [2f] J. L. Popot, E. A. Berry, D. Charvolin, C. Creuzenet, C. Ebel, D. M. Engelman, M. Flotenmeyer, F. Giusti, Y. Gohon, Q. Hong, J. H. Lakey, K. Leonard, H. A. Shuman, P. Timmins, D. E. Warschawski, F. Zito, M. Zoonens, B. Pucci, C. Tribet, *Cell Mol. Life Sci.* **2003**, *60*, 1559-74.