Tandem Facial Amphiphiles for Membrane Protein Stabilization

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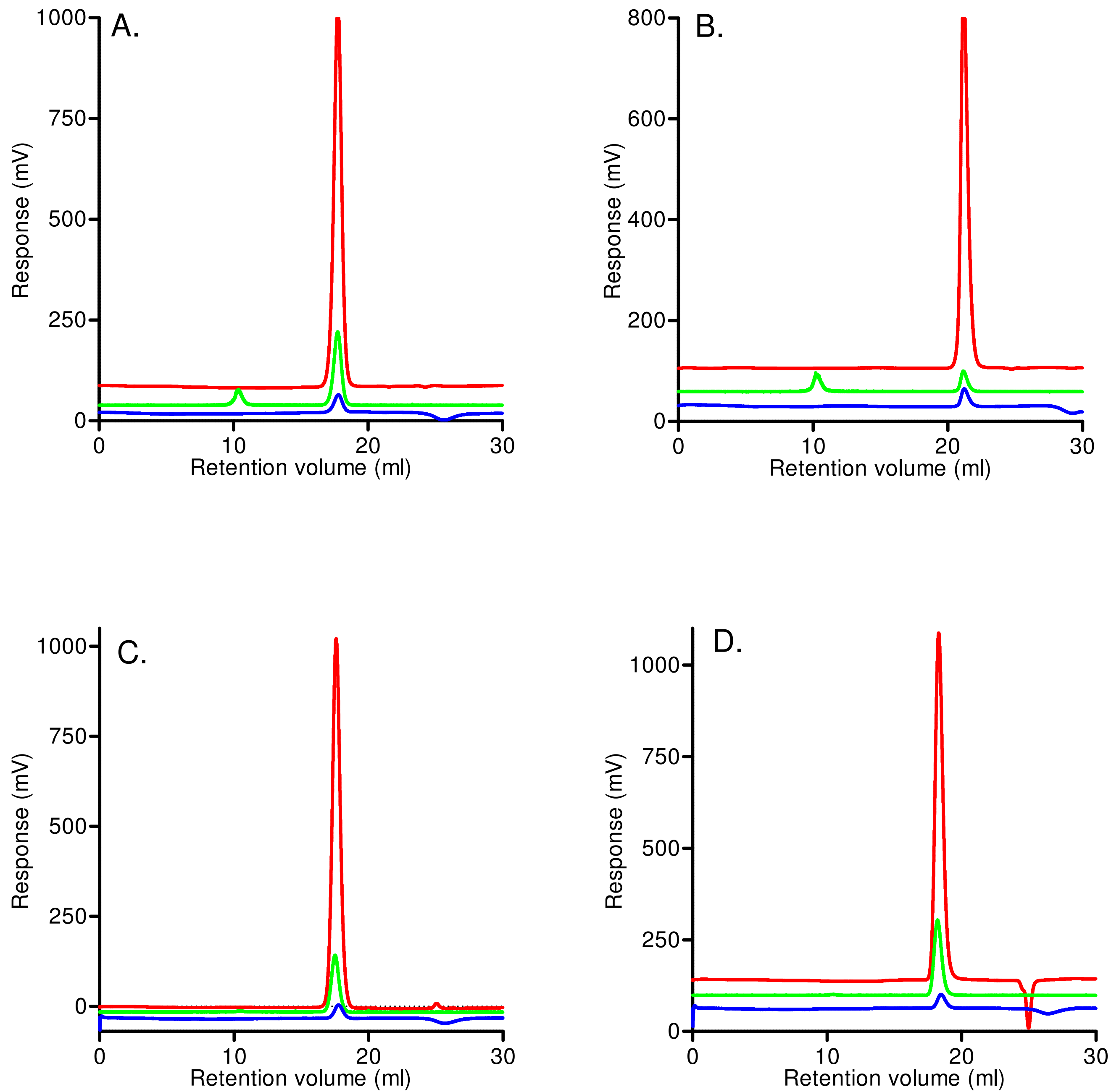
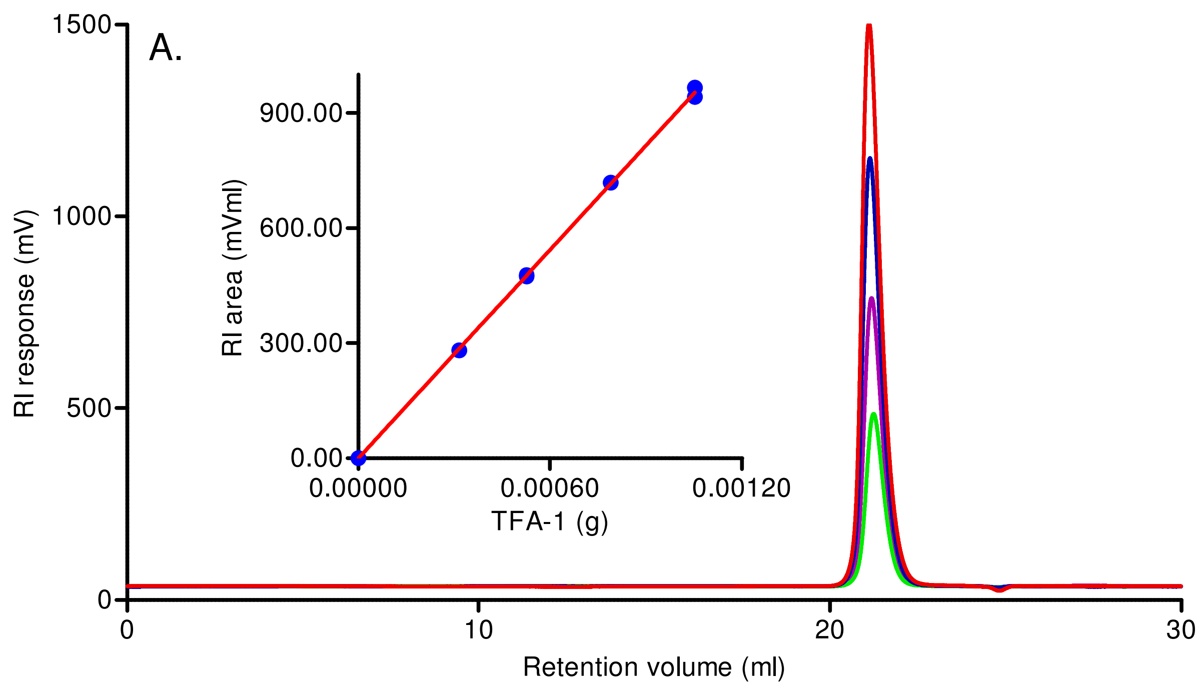


Figure S1. TDAgrams of 0.68 mg Ovalbumin (A) and 0.53 mg TFA-1 (B) in TFA buffer and 0.60 mg Ovalbumin (C) and 0.8mg DDM (D) in DDM buffer.



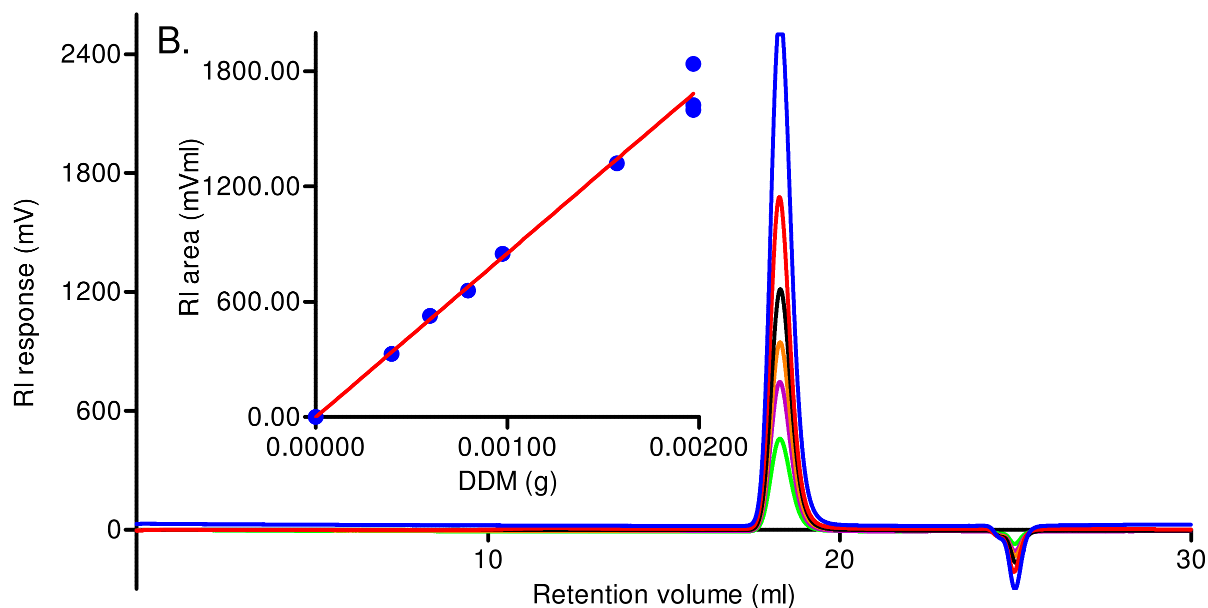


Figure S2. Measuring detergent dn/dc using TDA. Overlay of the RI traces used to calculate dn/dc (inset) from 7 different (0 to 1.05 mg; 4 shown) TFA-1 injections (A) and 10 different (0 to 1.97 mg; 6 shown) DDM injections (B).

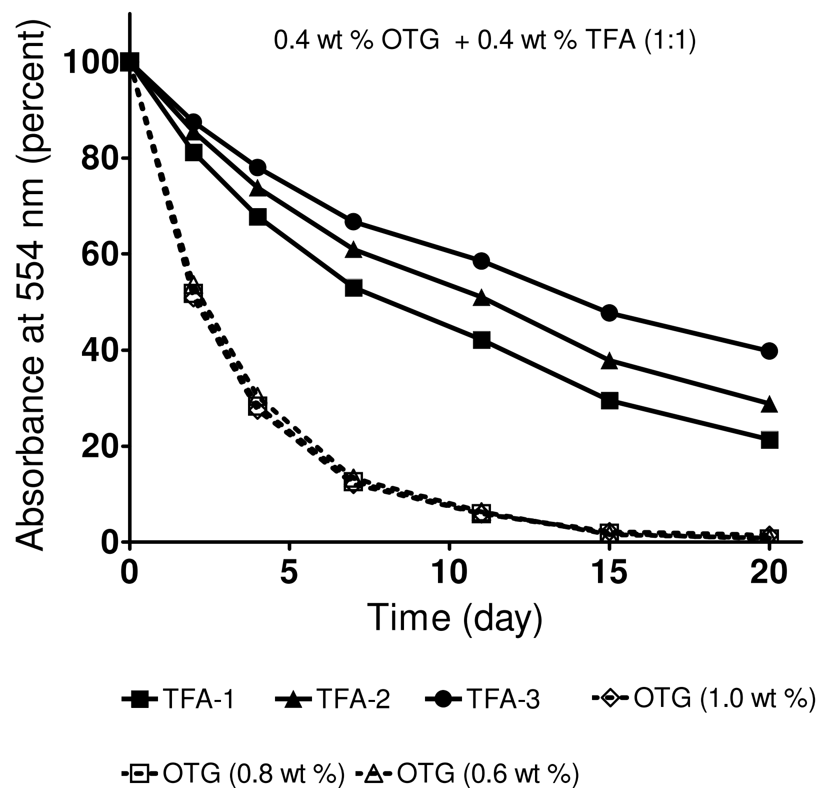


Figure S3. Time course of bR stability evaluated at room temperature. OTG was mixed with each TFA in a ratio of 1:1 or alone (0.6 wt %, 0.8 wt %, or 1.0 wt %). Absorbance at 554 nm was followed for the stability evaluation of the protein.

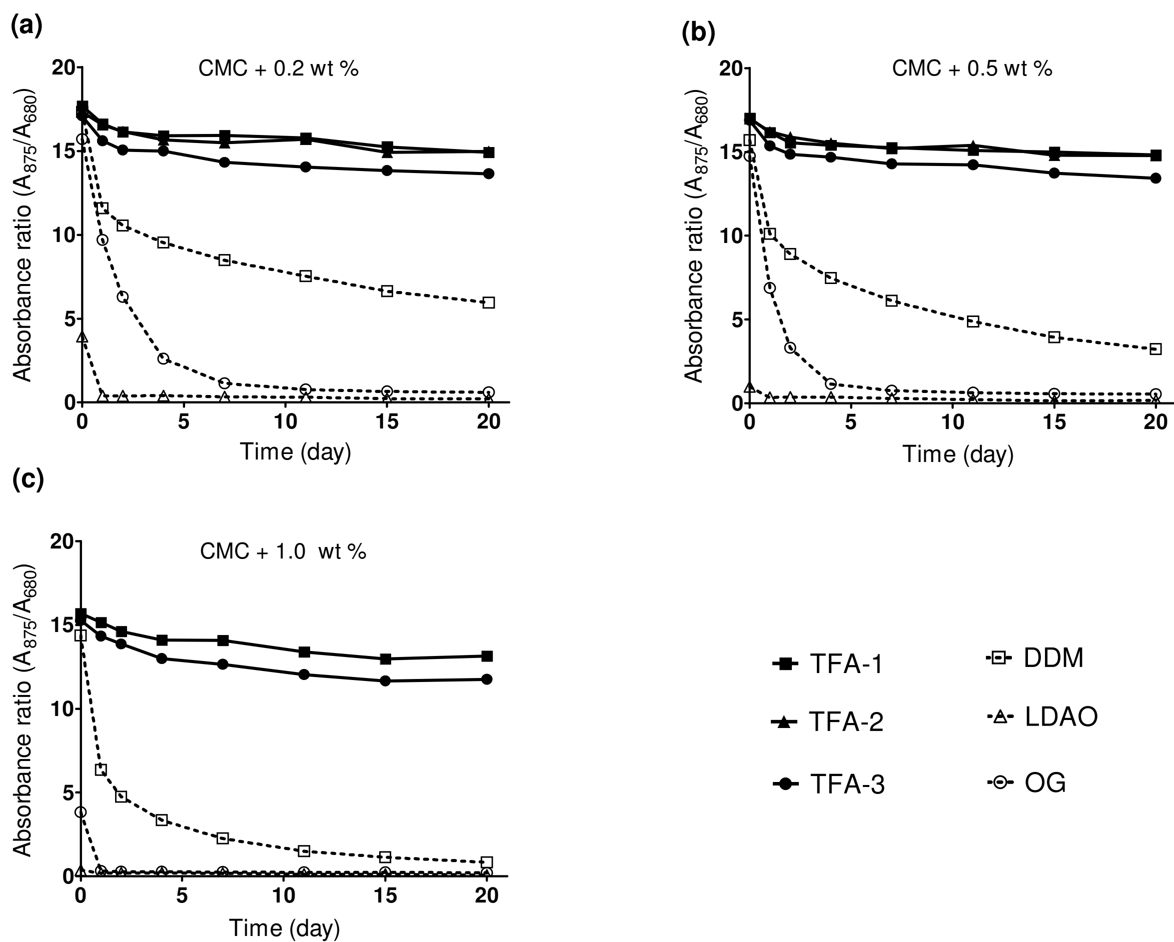


Figure S4. Time course of stability of *R. capsulatus* superassembly was evaluated at room temperature. Each TFA was used at three different concentrations (CMC + 0.2 wt %, CMC + 0.5 wt %, or CMC + 1.0 wt %). Absorbance ratio (A_{875}/A_{680}) was followed for the stability evaluation of the membrane protein.

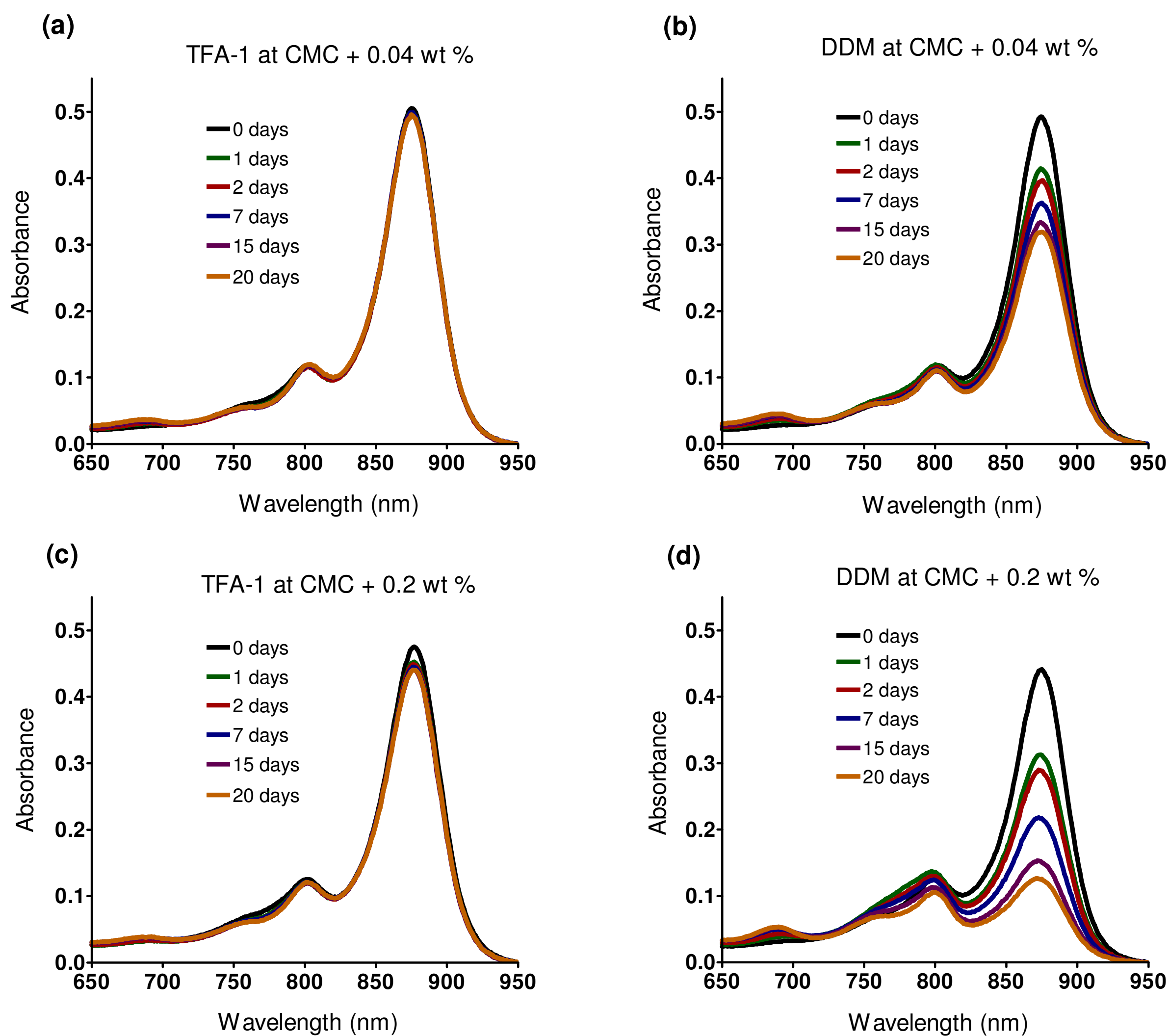


Figure S5*.* Absorbance spectrum of *R. capsulatus* superassembly in TFA-1 (a,c) and DDM (b,d) at room temperature. Each agent was used at two concentrations (CMC + 0.04 wt % and CMC + 0.2 wt %).

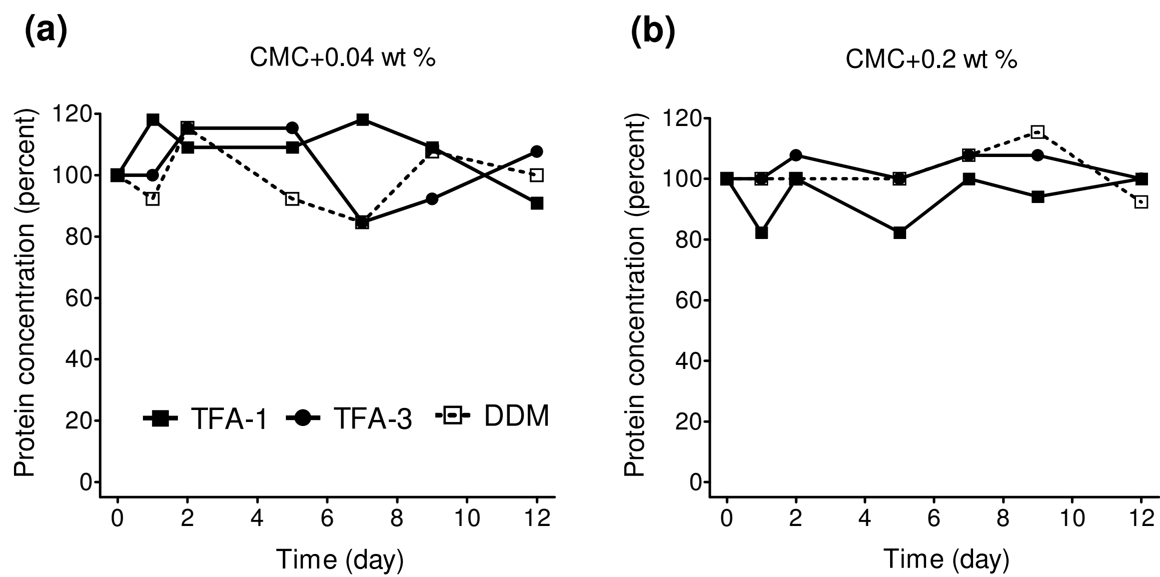
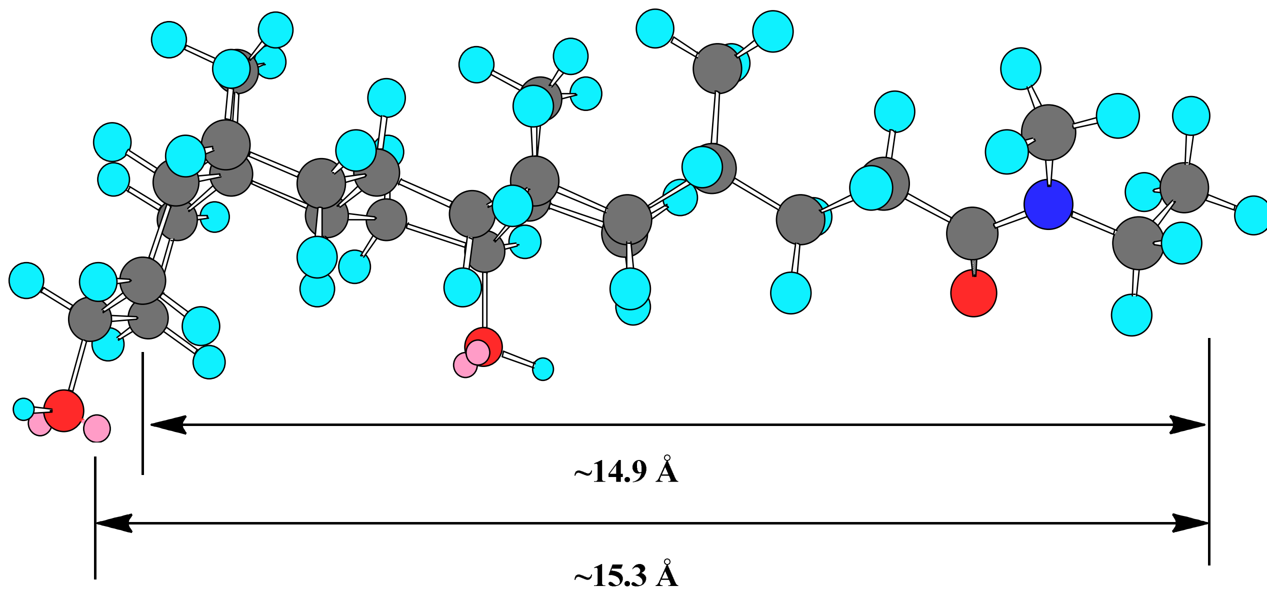


Figure S6. Long-term solubility of LeuT WT incubated in the presence of TFA-1, TFA-3, and DDM at (a) CMC + 0.04 wt % or (b) CMC + 0.2 wt % at room temperature.

An estimation of the length of the hydrophobic groups of the TFAs by Chem3D

The length of the hydrophobic group common to our TFA amphiphiles was estimated by measuring half of the chemical structure (as shown below) after MM2 energy minimization using the Chem3D program. The hydrophobic group adopts an extended conformation that is assumed to be representative of conformations populated by TFA amphiphiles when they interact with hydrophobic patches of membrane proteins. This approach gave a total TFA amphiphile length estimate of approximately 29 - 31 Å, which is similar to the length of the hydrophobic portion of a typical lipid bilayer (~30 Å).



Hydrodynamic radii (R_h) determination for TFAs and DDM using dynamic light scattering (DLS) measurements.

Each TFA and DDM was dissolved in distilled, deionized (DI) water to obtain 1 mL of a solution containing 0.5 wt % amphiphile. Each solution was filtered through a 0.02 µm filter for DLS measurements. A 100-mW, 532-nm laser (Compass 315M-100, Coherent, Santa Clara, CA) illuminated a temperature-controlled glass cell at 25°C that was filled with a refractive-index matching fluid (decahydronaphthalene, Fisher Scientific, Pittsburgh, PA). The scattered light was collected at an angle of 90° for about 30 minutes. R_h values were determined using the integrated dynamics software that analyzes the time scale of the scattered light intensity fluctuations by an autocorrelation function. The autocorrelation functions (ACFs) were obtained using a BI-9000AT digital autocorrelator (Brookhaven Instruments, Holtsville, NY). Measurements were repeated three times. The viscosity of pure water (0.89 cp at 25 °C) was used for all calculations, with the assumption that the low detergent concentration (0.5 wt %) does not have a significant effect. R_h values are expressed as mean ± SD ($n = 3$).

Micelle characterization of TFA-1 and DDM using triple detector analysis (TDA)

The on-line SEC triple detector array[1] (Viscotek Corporation, a Malvern Company) used for detergent analysis is composed of 1) a 660nm differential refractometer for quantifying the concentration C (mg/ml) for all molecules according to their specific refractive index increment dn/dc (ml/g) using Snell's Law of Refraction (RI signal = K_{RI} *C*(dn/dc)/RI_{Sol}), 2), a single right angle 90º static light scattering (RALS) detector for measuring the average mass M (Da) according to the Raleigh Light Scattering Equation for small molecules ($LS_{90º}$ signal = M_{avg} *RI_{sol}^2*C*(dn/dc) ^{2})/ K_{LS}*K_{opt}, where K_{opt} = $4*\pi^2/(\lambda^4*N_A)$, and 3) an absolute differential viscometer[2] for measuring differential pressure (DP) and calculating intrinsic viscosity (IV; dL/g) according to Newtonian Viscosity (liquid layers) applied to tubes using Poiseulle's Law (DP signal = IV*C) and for calculating the radius of hydration Rh (nm) according to Einstein's volume of hydration equation for hard spheres ($R_h = [(3/10\pi)*IV*M/N_A]^{1/3}$). The IV represents molecular shape and for proteins it can vary from 0.017 dl/g for globular shapes to 0.355 dl/g for elongated shapes to 0.499 dl/g for complete denaturation[3, 4, 5]. The chromatography pump, injector, SEC column (0.75 x 60cm TSK G3000 plus guard column), and TDArray are placed in a 4ºC cold box, while the buffer (degassed for more than 1 hr with stir prior use) is at room temperature (RT) to allow it to be run through a RT on-line degasser, which then runs though 3 ft of 4ºC stainless steel tubing before entering the SEC pump, injector and column. The extensive degassing insures an accurate DP measurement. With this configuration, the temperature at the detectors is 6ºC. TDA buffer was 20mM Hepes, 150mM NaCl, pH 7.3 and either 0.1mM TFA-1 or 1mM DDM. The refractive index of the solvent RI_{Sol} was measured at 10ºC using an Anton Paar Abbemat temperature controlled refractometer at 589.2nm (generously on loan from Jay Skovbjerg at Anton Paar). Triple detection data at every 5 Hz data point throughout the complete SEC peak are used for all analysis.

For calibration, the detector response factors K_{RI} and K_{LS} were measured using Ovalbumin Fraction VII (Sigma). Prior to calibration, ovalbumin was first purified by SEC (280nm detection) at 4ºC to remove small populations of dimers and aggregates, and to insure Gaussian detector peaks for TDA calibration. Ovalbumin was solubilized at 26mg/ml in TDA buffer at 4ºC for 10 min and clarified by centrifugation (10 min 15,000 x g); collecting the top 30% of the SEC elution peak from 500µl injections yielded monodisperse ovalbumin at 5-7 mg/ml. Ovalbumin mass is 44.3kDa (Sigma Product Information sheet and references therein) with a 0.701 dA_{280nm} /dc [6] and 0.187 dn/dc[7, 8]. The absolute error of the detector response factors from 4 measurements for each detergent was ≤ 0.1% for TFA-1 buffer and ≤ 0.2% for DDM buffer. Examples of TDA SEC profiles (TDAgrams) are shown in Figure 1. RI data and analysis used to measure detergent dn/dc are shown in Figure 2.

Protein stability evaluation

Stabilization assay for bacteriorhodopsin (bR)

The general procedure was followed according to the reported protocol[9]. Frozen aliquots of purple membranes containing bR at 184 μM were thawed and solubilzed by incubation with octylthioglucoside (OTG) for 24 hr at 4°C in a dark room. For the solubilization, OTG (CMC = 0.28 wt %) was used at 2.0 wt % in 10 mM sodium phosphate (pH 6.9). Subsequently the solubilized material was separated from the membrane debris in an ultracentrifuge at 200,000 *x g* at 4°C for 20 min. The supernatant from the spin was collected and transferred into individual TFA solutions to give final detergent/amphiphile concentration of OTG : TFA = 0.4 wt % : 0.4 wt % (1:1) or 0.2 wt % : 0.8 wt % (1:4). BR stability in each TFA solutions was monitored by measuring absorbance at 554 nm for 20 days.

Stabilization assay for *R. capsulatus* superassembly

The general procedure was followed according to the reported protocol[10]. Briefly, specialized photosynthetic membranes were prepared from an engineered strain of *Rhodobacter* (*R.*) *capsulatus*, U43[pUHTM86Bgl], lacking the LHII light-harvesting complex and used as the starting material. The solubilization and purification of LHI-RC superassembly were started by thawing, homogenizing, and equilibrating frozen aliquots of *R. capsulatus* membranes at 32˚C for 30 min. Addition of DDM at 1 wt % concentration to 1 mL aliquots of the membranes was followed by incubation of the membrane samples at 32°C for 30 min. Subsequently, the solubilized material was collected and transferred into a new microcentrifuge tube containing Ni-NTA resin (Qiagen, Inc.; Valencia, CA; pre-equilibriated and stored in an equal volume of buffer containing 10 mM Tris, pH 7.8, and 100 mM NaCl) after an ultracentrifuge at 315,000 *x g* at 4°C for 30 min. The tubes were then incubated and inverted for 1 h at 4°C for binding. Resin-retaining spin columns (e.g., emptied His SpinTrap™ columns; GE Healthcare) were inserted into a 2 mL microcentrifuge tube and the samples were then loaded onto the column. Samples were washed twice with 0.5 mL of binding buffer (a pH 7.8 Tris solution containing DDM at 1xCMC). The protein was collected by eluting with two 0.25 mL elution buffer aliquots (this buffer was identical to binding buffer with the addition to 1 M of imidazole; the pH of each solution was checked and readjusted to pH = 7.8). Small aliquots (0.05 mL) of the purified protein solutions were transferred to 0.95 mL individual amphiphiles/detergents solutions at the designated concentrations (CMC+0.04 wt %, CMC+ 0.2 wt %, CMC+ 0.5 wt % and CMC + 1.0 wt %) and incubated at room temperature. UV-Vis spectra of these solutions were measured in regular intervals. Degradation of the material could be monitored with the 875 nm/680 nm absorbance ratio, which decreased with time and sample integrity as the dominant 875 nm absorption of intact LHI disappeared and a 680 nm band, indicating the presence of unbound, oxidized cofactors, appeared.

Thermal stability assay for CytBO3

The thermal stability assay method was performed as described[11] with the following minor modifications. CPM (Invitrogen) dye aliquots, stored in DMSO (Sigma), were diluted in dye buffer [20 mM Tris (pH 7.5), 150 mM NaCl, 0.03% DDM, 5 mM EDTA]. All the detergent/amphiphiles were used at CMC + 0.04 wt % in test buffer [20 mM Tris (pH 7.5), 150 mM NaCl]. Greiner 96-well plates were used, and the volume of selected buffer solutions was 150µL. The test protein (10 mg/ml) was diluted in the assay buffer solutions (1:150) in Greiner 96-well plates, and 3 µL of diluted CPM dye was added to each test condition. The reaction was monitored for 130 min at a fixed temperature of 40°C using a microplate spectrofluorometer set at an excitation wavelength of 387 nm and an emission wavelength of 463 nm. Readings were taken every 5 min after automatic agitation of the plate. Relative maximum fluorescence count was used to calculate percentage of relative folded protein remaining after 130 minutes at 40°C. Relative unfolding profiles of proteins were plotted against time using GraphPad Prism using the one-phase decay equation.

LeuT solubility and functionality assay.

The wild type of the leucine transporter (LeuT WT) from *Aquifex aeolicus* was expressed in *Escherichia coli* essentially as described[12]. The pET16b-LeuT WT-8His plasmid was kindly provided by Dr. Eric Gouaux, Vollum Institute, Portland Oregon. LeuT WT was extracted from isolated bacterial membranes solubilized with 1 % DDM, bound to Ni^{2+}-affinity Chelating Sepharose Fast Flow resin (GE Healthcare, USA), and eluted in buffer consisted of 20 mM Tris-HCl (pH 8.0), 1 mM NaCl, 199 mM KCl, 0.05% DDM and 300 mM imidazole. Subsequently, selected LeuT WT fractions were pooled, aliquoted and diluted in the above-mentioned buffer without DDM, but containing TFA-1 or TFA-3 in a final concentration of CMC + 0.04 wt % or CMC + 0.2 wt %, respectively. Alternatively, as control, DDM was used at the above-mentioned final concentrations. After incubation at the room temperature, at the indicated time points, samples were centrifuged and the protein concentration was determined by absorbance measurements at 280 nm. Concomitantly, [^{3}H]-Leu binding was determined using scintillation proximity assay (SPA)[13], with the reaction mixture consisted of 5 µL from the respective samples, 33.3 nM [^{3}H]-Leu (PerkinElmer, USA) and copper chelate (His-Tag) YSi beads (GE Healthcare). NaCl and the tested compounds at the above-mentioned concentrations. For each time point, double determination of [^{3}H]-Leu binding was performed using MicroBeta liquid scintillation counter (PerkinElmer). Normalized results are expressed as mean ± SEM (n = 2).

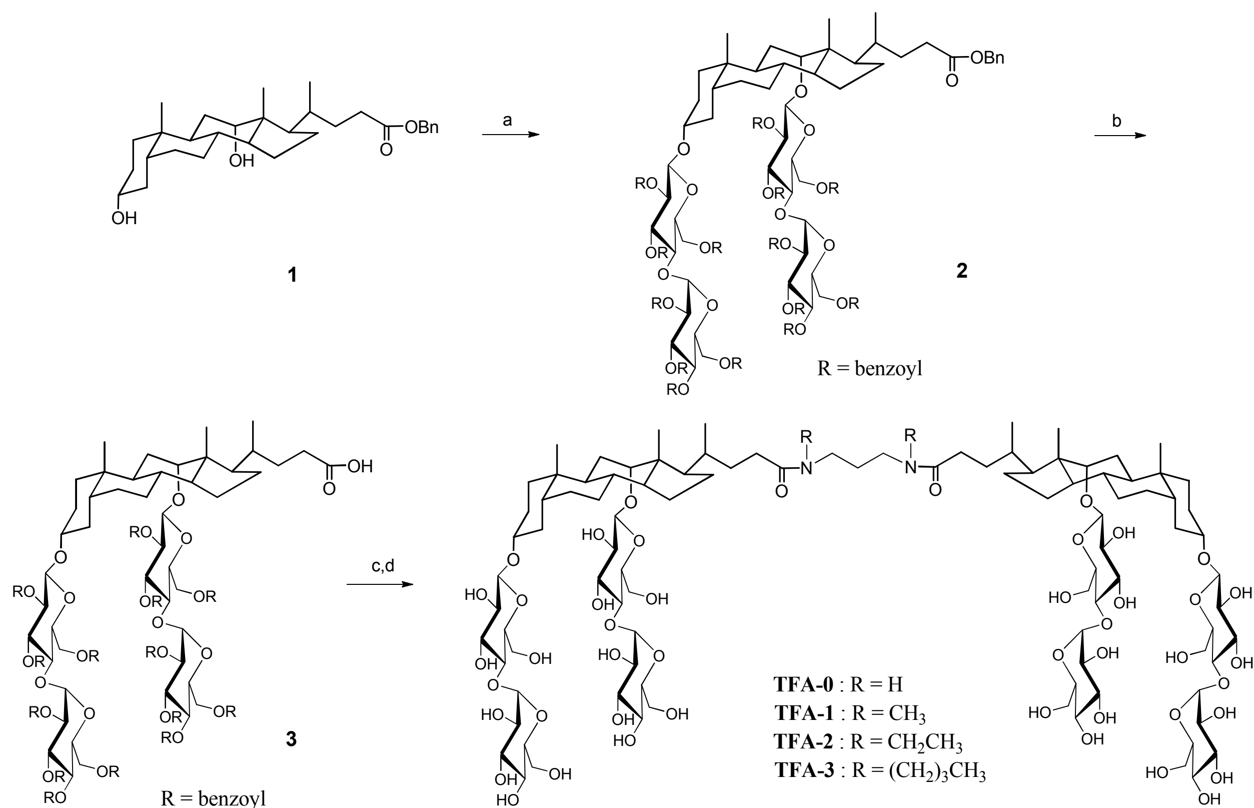
Temperature ramp stability assay for β_2AR-T4L.

Sf9 insect cell cultures expressing baculovirus encoded β_2AR-T4L were solubilized and purified in DDM as previously described[14]. Briefly, the receptor was purified by M1 FLAG antibody (Sigma) chromatography followed by alprenolol-Sepharose affinity chromatography. The receptor was immobilized in a second M1 chromatography step and

washed extensively in buffer (0.1% DDM, 100 mM NaCl, 20 mM HEPES, pH 7.5) containing 30 μM carazolol to exchange bound alprenolol. The eluted carazolol-bound receptor was dialyzed against buffer containing 1 μM carazolol and spin concentrated to 7 mg/ml (≈ 140 μM) using a 100 kDa molecular weight cut-off Vivaspin (Vivascience) concentrator. For stability measurements the carazolol-bound β_2AR-T4L was diluted below the CMC for DDM by adding 3 μL of the concentrated receptor in a quartz cuvette containing 600 μL buffer (100 mM NaCl, 20 mM HEPES, pH 7.5) with TFA-1 and TFA-3. The cuvette was placed in a Spex FluoroMax-3 spectrofluorometer (Jobin Yvon Inc.) under *Peltier* temperature control. Fluorescence emission from carazolol was obtained following 5 min incubations from 25 to 85°C in twelve continuous 5°C increments. Emission was obtained from 335 to 400 nm with excitation set at 325 nm using an integration time of 0.3 s nm^{-1} and a 1 nm bandpass for both excitation and emission. The 341:356 nm peak ratio was calculated using Microsoft Excel and graphed using GraphPad Prism software.

Amphiphile Synthesis

Synthetic Scheme



(a) perbenzoylated maltosylbromide, AgOTf, CH_2Cl_2, -45°C → room temperature, 3 hr (b) $NH_4^+CO_2^-$, Pd/C, EtOAc/MeOH, room temperature, 1 hr (c) EDC • HCl, HOBt, room temperature , 2 days (d) NaOMe, MeOH, room temperature, 4 hr.

Compound **2**

This reaction was performed according to a literature method[15] with slight modification. A mixture of **1**[16] (0.83 g, 1.7 mmol.), AgOTf (0.97 g, 3.8 mmol), 2,4,6-collidine (0.39 g, 2.9 mmol) in anhydrous CH_2Cl_2 (40 mL) was stirred at -45°C. A solution of perbenzoylated maltosylbromide (4.3 g, 3.8 mmol) in CH_2Cl_2 (40 mL) was added dropwise over 0.5 h to this suspension. Stirring was continued for 0.5 h at -45°C, and then the reaction mixture was rapidly warm to 25 °C and left stirring for 3 h. After completion of reaction (as detected by TLC), pyridine was added to the reaction mixture, and it was diluted with CH_2Cl_2 (40 mL) before being filtered over celite. The filtrate was washed successively with a 1 M aqueous $Na_2S_2O_3$ solution (40 mL), a 0.1 M aqueous HCl solution (40 mL), and brine (2 x 40 mL). Then the organic layer was dried with anhydrous Na_2SO_4 and the solvents were removed by rotary evaporation. The residue was purified by silica gel column chromatography (EtOAc/hexane) providing desired product (**2**) as a glassy solid(3.2 g, 72%). **^{1}H NMR** (300 MHz,

$CDCl_3$): δ 8.13-8.05 (m, 4H), 8.05-7.92 (m, 12H), 7.90-7.84 (m, 8H), 7.62-7.82 (m, 22H), 7.62-7.05 (m, 99H), 6.10 (q, *J* = 10.1Hz, 4H), 5.84-5.60 (m, 12H), 5.40-5.22 (m, 8H), 5.08-4.82 (m, 8H), 4.75-4.18 (m, 20H), 4.18-4.04 (m, 4H), 4.04-3.92 (m, 2H), 3.76 (br s, 2H), 3.55-3.40 (m, 2H), 2.05-1.95 (m, 2H), 1.95-1.80 (m, 6H), 1.80-1.00 (m, 52H), 1.00-0.72 (m, 28H), 0.70-0.60 (m, 10H), 0.60-0.45 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 173.6, 166.4, 166.1, 165.8, 165.7, 165.6, 165.4, 165.2, 165.1, 164.9, 133.6, 133.5, 133.4, 133.3, 133.2, 133.1, 130.2, 130.1, 130.0, 129.9, 129.8, 129.6, 129.2, 129.1, 129.0, 128.9, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 102.7, 100.1, 96.9, 96.8, 85.4, 81.5, 77.4, 75.2, 75.3, 74.8, 74.1, 73.2, 72.8, 71.4, 70.9, 70.3, 70.2, 69.5, 69.4, 69.3, 69.2, 66.0, 62.9, 53.6, 48.0, 47.1, 46.8, 42.5, 36.4, 35.5, 35.3, 34.6, 34.5, 34.0, 32.2, 29.5, 27.7, 26.1, 24.0, 23.4, 17.2, 12.6 ; **MS (MALDI-TOF)**: calcd. for $C_{153}H_{142}O_{38}$ $[M+Na]^+$ 2611.7, found 2611.4.

Compound **3**

This reaction was performed according to a literature method[17] with slight modification. Compound **2** (0.85 g, 0.33 mmol), 10% Pd/C (0.17 g) and ammonium formate (0.21 g, 3.28 mmol) were suspended in MeOH: EtOAC (15 mL : 9 mL). The solution was stirred for 1 h at 25 °C. The solution was filtered through celite, which was rinsed with MeOH, and the solution was evaporated to give desired prouduct (**3**) as a glassy solid (0.80 g, 98%). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.13-8.05 (m, 4H), 8.05-7.92 (m, 12H), 7.90-7.84 (m, 8H), 7.62-7.82 (m, 22H), 7.62-7.05 (m, 94H), 6.10 (q, *J* = 10.1Hz, 4H), 5.84-5.60 (m, 12H), 5.40-5.22 (m, 8H), 5.08-4.82 (m, 8H), 4.75-4.18 (m, 20H), 4.18-4.04 (m, 4H), 4.04-3.92 (m, 2H), 3.76 (br s, 2H), 3.55-3.40 (m, 2H), 2.05-1.95 (m, 2H), 1.95-1.80 (m, 6H), 1.80-1.00 (m, 52H), 1.00-0.72 (m, 28H), 0.70-0.60 (m, 10H), 0.60-0.45 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 178.9, 166.4, 166.1, 166.0, 165.9, 165.8, 165.4, 165.3, 165.2, 164.9, 133.5, 133.4, 133.3, 133.2, 133.0, 130.2, 130.1, 130.0, 129.9, 129.8, 129.7, 129.6, 129.5, 129.4, 129.3, 129.2, 129.1, 129.0, 128.7, 128.6, 128.5, 128.4, 128.3, 128.2, 128.0, 127.9, 110.9, 102.7, 100.1, 96.9, 96.8, 85.3, 81.5, 77.4, 75.6, 75.3, 74.8, 74.2, 73.2, 73.1, 72.8, 71.5, 71.0, 70.3, 70.2, 69.5, 69.4, 69.3, 69.2, 64.2, 62.9, 48.0, 47.1, 46.9, 42.5, 36.4, 35.6, 34.6, 34.5, 34.0, 31.8, 29.4, 27.8, 27.7, 27.6, 23.5, 17.3, 12.7 ; **MS (MALDI-TOF)**: calcd. for $C_{146}H_{136}O_{38}$ $[M+Na]^+$ 2521.6, found 2521.1.

TFA-0

Compound **3** (900 mg, 0.36 mmol), 1,3-diaminopropane (15 mg, 0.17 mmol), 1-hydroxybenzotriazole monohydrate (HOBt) (58 mg, 0.43 mmol) was dissolved in anhydrous DMF (30 mL). 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochoride (EDC • HC1) (83 mg, 0.43 mmol) was added in small portions at 0°C and the resulting solution left stirring at room temperature for 48 h. The solution was taken up with EtOAc (100 mL) and was washed successively with a 1 M aqueous $NaHCO_3$ solution (100 mL), a 0.1 M aqueous HCl solution (100 mL) and brine (2 x 100 mL). Then the organic layer was dried with anhydrous Na_2SO_4 and the solvent was removed by rotary evaporation, and used in next reaction without further purification. The resulting *O*-benzoylated compounds were dissolved in MeOH and

then treated with the required amount of a methanolic solution of 0.5 M NaOMe such that the final concentration of NaOMe was 0.05 M. The reaction mixture was left stirring for 6 h at room temperature, and then neutralized with Amberlite IR-120 (H^+ form) resin. The resin was removed by filtration and washed with MeOH and solvent was removed from the combined filtrate *in vacuo*. The residue was purified by silica gel column chromatography (MeOH/CH_2Cl_2). Further purification carried out by recrystallization using CH_2Cl_2/MeOH/diethyl ether afforded fully de-*O*-benzoylated product (**TFA-0**) as a white solid (0.69 g, 90%). **^{1}H NMR** (300 MHz, CD_3OD): δ 5.22 (d, *J* = 3.8 Hz, 2H), 5.19 (d, *J* = 3.8 Hz, 2H), 4.45 (d, *J* = 7.6 Hz, 2H), 4.39 (d, *J* = 7.6 Hz, 2H), 3.96-3.78 (m, 14H), 3.78-3.58 (m, 20H), 3.58-3.45 (m, 10H), 3.45-3.15 (m, 12H), 2.36-2.06 (m, 8H), 2.04-1.82 (m, 10H), 1.82-1.53 (m, 14H), 1.53-1.35 (m, 12H), 1.35-1.05 (m, 14H), 1.05-0.88 (m, 8H), 0.74 (s, 6H) 7; **^{13}C NMR** (75 MHz, CD_3OD): δ 177.5, 106.7, 103.1, 102.8, 102.4, 86.2, 81.8, 81.5, 80.6, 78.3, 78.0, 76.7, 75.5, 75.2, 74.9, 74.8, 74.3, 71.7, 71.6, 62.9, 62.4, 46.3, 43.9, 37.9, 37.6, 37.5, 36.6, 35.8, 35.6, 35.3, 33.8, 33.3, 30.4, 29.3, 28.6, 28.5, 28.4, 27.8, 25.2, 23.9, 18.2, 13.0; **MS (MALDI-TOF)**: calcd. for $C_{99}H_{166}$ N_2O_{46} $[M+Na]^+$ 2142.0, found 2142.1.

TFA-1: *N,N'*-dimethyl-1,3-propanediamine was used intead of 1,3-diaminopropane in the synthesis of TFA-0. **^{1}H NMR** (300 MHz, CD_3OD): δ 5.22 (d, *J* = 3.8 Hz, 2H), 5.19 (d, *J* = 3.8 Hz, 2H), 4.45 (d, *J* = 7.6 Hz, 2H), 4.40 (d, *J* = 7.6 Hz, 2H), 3.96-3.78 (m, 14H), 3.78-3.58 (m, 20H), 3.58-3.20 (m, 22H), 3.10 (d, *J* = 10.3 Hz, 4H), 2.95 (d, *J* = 10.7 Hz, 2H), 2.36-2.06 (m, 8H), 2.04-1.82 (m, 10H), 1.82-1.53 (m, 14H), 1.53-1.35 (m, 12H), 1.35-1.05 (m, 14H), 1.05-0.88 (m, 8H), 0.76 (s, 6H); **^{13}C NMR** (75 MHz, CD_3OD): δ 176.9, 176.7, 176.6, 106.8, 103.0, 102.7, 102.4, 86.2, 81.6, 81.4, 80.6, 78.2, 77.9, 76.7, 75.4, 75.2, 74.9, 74.8, 74.3, 71.6, 63.2, 62.9, 62.4, 46.7, 46.3, 43.8, 37.6, 37.5, 37.4, 36.6, 36.4, 35.7, 35.6, 35.3, 33.9, 32.6, 31.2, 29.3, 28.7, 28.5, 27.8, 25.3, 23.9, 18.4, 18.3, 13.0 ; **MS (MALDI-TOF)**: calcd. for $C_{101}H_{170}N_2O_{46}$ $[M+Na]^+$ 2170.1, found 2170.0.

TFA-2: *N,N'*-diethyl-1,3-propanediamine was used intead of 1,3-diaminopropane in the synthesis of TFA-0. **^{1}H NMR** (300 MHz, CD_3OD): δ 5.22 (d, *J* = 3.8 Hz, 2H), 5.19 (d, *J* = 3.8 Hz, 2H), 4.45 (d, *J* = 7.6 Hz, 2H), 4.40 (d, *J* = 7.6 Hz, 2H), 3.96-3.78 (m, 14H), 3.78-3.58 (m, 20H), 3.58-3.20 (m, 26H), 2.36-2.06 (m, 8H), 2.04-1.82 (m, 10H), 1.82-1.53 (m, 14H), 1.53-1.35 (m, 12H), 1.35-1.05 (m, 14H), 1.05-0.88 (m, 8H), 0.76 (s, 6H); **^{13}C NMR** (75 MHz, CD_3OD): δ 176.5, 176.4, 176.2, 106.7, 103.0, 102.8, 102.5, 98.6, 86.2, 81.6, 81.4, 80.6, 78.3, 78.0, 76.7, 75.5, 75.2, 74.9, 74.8, 74.3, 74.2, 71.7, 71.6, 63.2, 62.9, 62.4, 55.3, 48.3, 48.1, 46.5, 44.4, 43.9, 37.6, 36.6, 35.8, 35.6, 35.3, 29.4, 28.7, 28.5, 28.4, 27.8, 25.3, 23.9, 18.5, 18.4, 18.3, 14.8, 14.7, 13.4, 13.1, 13.0; **MS (MALDI-TOF)**: calcd. for $C_{103}H_{174}N_2O_{46}$ $[M+Na]^+$ 2198.1, found 2198.0.

***N,N'*-dibutyl-1,3-propanediamine**

Butyric acid (1.8 g, 19.4 mmol), 1,3-diaminopropane (0.60 g, 8.1 mmol), 1-hydroxybenzotriazole monohydrate (HOBt) (2.4 g, 17.8 mmol) was dissolved in anhydrous DMF (30 mL). 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide

hydrochoride (EDC • HC1) (3.4 g, 17.8 mmol) was added in small portions at 0°C and the resulting solution left stirring at room temperature for 15 h. After evaporation of DMF, the solution was taken up with $CHCl_3$ (200 mL) and was washed successively with a 1 M aqueous $NaHCO_3$ solution (30 mL), a 0.1 M aqueous HCl solution (30 mL) abd brine (30 mL). Then the organic layer was dried with anhydrous Na_2SO_4 and the solvent was removed by rotary evaporation, The resulting residue was precipitated using ether and used in next reaction without further purification. To the precipitate dissolved in THF (50mL) $LiAlH_4$ (0.40 g, 10.6 mmol) was added slowly at 0°C. The mixture was refluxed overnight, quenched carefully with 0.4 mL of water, 0.4 mL of 15% NaOH and 1.2 mL of water. After filtration on celite, the filrate was evaporated , dissolved in DCM (100 mL) and extracted with 1N NaOH solution (50 mL). The organic layer dried with anhydrous Na_2SO_4. The residue was purified by silica gel column chromatography ($MeOH/CH_2Cl_2$) providing ***N,N'*-dibutyl-1,3-propanediamine** as an oil (0.90 g, 52% (two steps)). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 2.67 (t, *J* = 7.0 Hz, 4H), 2.59 (t, *J* = 7.0 Hz, 4H), 1.69 (quin, *J* = 7.0 Hz, 2H), 1.52-1.39 (m, 4H), 1.39-1.24 (m, 4H), 0.91 (t, *J* = 7.2 Hz, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 50.1, 48.9, 32.5, 30.7, 20.7, 14.2; **HRMS (ESI)**: calcd. for $C_{11}H_{26}N_2[M+H]^+$ 187.2169, found 187.2170.

TFA-3: *N,N'*-dibutyl-1,3-propanediamine was used intead of 1,3-diaminopropane in the synthesis of TFA-0. **^{1}H NMR** (300 MHz, CD_3OD): 5.22 (d, *J* = 3.8 Hz, 2H), 5.19 (d, *J* = 3.8 Hz, 2H), 4.50-4.37 (m, 4H), 4.00-3.78 (m, 14H), 3.78-3.56 (m, 20H), 3.56-3.18 (m, 26H), 2.54-2.10 (m, 8H), 2.04-1.85 (m, 10H), 1.85-1.55 (m, 16H), 1.55-1.23 (m, 22H), 1.23-1.06 (m, 10H), 1.06-0.86 (m, 14H), 0.77 (s, 6H); **^{13}C NMR** (75 MHz, CD_3OD): δ 176.7, 176.5, 176.4, 106.8, 106.7, 103.0, 102.8, 102.4, 86.1, 81.6, 81.4, 80.6, 78.3, 77.9, 76.7, 75.6, 75.4, 75.2, 74.9, 74.8, 74.3, 71.7, 71.6, 63.2, 62.9, 62.4, 62.4, 55.8, 46.9, 46.5, 44.9, 43.8, 37.6, 37.4, 36.6, 35.7, 35.6, 35.3, 33.3, 33.2, 32.6, 32.5, 31.1, 29.4, 28.7, 28.5, 28.4, 27.8, 25.3, 23.9, 21.4, 21.3, 21.2, 18.5, 18.4, 18.3, 14.5, 14.4, 13.1, 13.0; **MS (MALDI-TOF)**: calcd. for $C_{107}H_{182}N_2O_{46}$ $[M+Na]^+$ 2282.2, found 2282.2.

References

1. Gatta, A. L.; Rosa, M. D.; Marzaioli, L.; Busico, T.; Schiraldi, C. A complete hyaluronan hydrodynamic characterization using a size exclusion chromatography–triple detector array system during in vitro enzymatic degradation. *Anal. Biochem.* **404**, 21–29 (2010) .

2. Haney, M. A. The differential viscometer. I. A new approach to the measurement of specific viscosities of polymer solutions. *J. Appl. Polym. Sci.* **30**, 3023-3036 (1985).

3. Scheraga H. A.; Mandelkern, L. Consideration of the hydrodynamic Properties of Proteins, *J. Chem. Phys*. **75**, 179-184 (1953).

4. Dutta, P. K.; Hammons, K.; Willibe, B.; Haney, M. A. Analysis of protein denaturation by high-performance continuous differential viscometry. *J. Chromatogr.* **536**, 113-121 (1991).

5. Chenal, A.; Guijarro, J. I.; Raynal ,B.; Delepierre, M.; Ladant, D. RTX calcium binding motifs are intrinsically disordered in the absence of calcium: implication for protein secretion. *J. Biol. Chem.* 16, **284**(3), 1781-1789 (2009) .

6. Pace, C. N.; Vajdos, F.; Fee, L.; Grimsley, G.; Gray, T. How to measure and predict the molar absorption coefficient of a protein. *Prot. Sci.* **4**(11), 2411-2423 (1995).

7. Maezawa, S.; Hayashi. Y.; Nakae, T.; Ishii. J.; Kameyama, K.; Takagi. T. Determination of molecular-weight of membrane-proteins by the use of low-angle laser-light scattering combined with high-performance gel chromatography in the presence of a non-ionic surfactant. *Biochem. Biophys. Acta* **747**, 291-297 (1983).

8. Hayashi , Y.; Matsui, H.; Takagi, T. Membrane protein molecular weight determined by low-angle laser light-scattering photometry coupled with high-performance gel chromatography. *Methods Enzymol.* **172**, 514-528 (1989).

9. Bazzacco, P.; Sharma, K. S.; Durand, G.; Giusti, F.; Ebel, C.;Popot, J.-L.; Pucci, B. *Biomacromolecules* **10**, 3317-3326 (2009).

10. Chae, P. S.; Wander, M. J.; Bowling, A. P.; Laible, P. D.; Gellman, S. H. Glycotripod amphiphiles for solubilization and stabilization of a membrane protein superassembly: importance of branching in the hydrophilic portion. *ChemBioChem* **9**, 1706-1709 (2008).

11. Alexandrov, A.; Mileni, M.; Chien, E.Y.; Hanson, M.A.; Stevens, R.C. Microscale fluorescent thermal stability assay for membrane proteins. *Structure* **16**, 351-359 (2008).

12. Deckert, G. *et al*. The complete genome of the hyperthermophilic bacterium *Aquifex aeolicus*. *Nature* **392**, 353-358 (1998).

13. Quick, M.; Javitch J. A. Monitoring the function of membrane transport proteins in detergent-solubilized form. *Proc. Natl. Acad. Sci. USA* **104**, 3603-3608 (2007).

14. Rosenbaum, D.M. *et al*. GPCR engineering yields high-resolution structural insights into β_2-adrenergic receptor function. *Nature* **318**, 1266-1273 (2007).

15. Ashton, P. R.; Boyd, S. E.; Brown, C. L.; Jayaraman, N.; Nepogodiev, S . A.; Stoddart, J. F. A convergent synthesis of carbohydrate-containing dendrimers. *Chem. Eur. J*. **2**, 1115-1128 (1996).

16. Vijayalakshmi, N.; Maitra, U. Multiple chromophore labeled novel bile acid dendrimers for light harvesting. *Macromolecules* **39**, 7931-7940 (2006).

17. Roy, R. ; Tropper, F. D. Carbohydrate protein interactions. Syntheses of agglutination inhibitors of wheat germ by phase transfer catalysis. *Can. J. Chem.* **69**, 817-821 (1991).