CHEMISTRY
A EUROPEAN JOURNAL

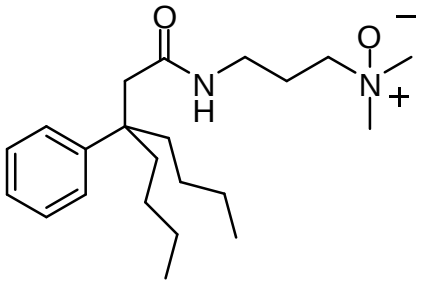
Supporting Information

Novel Tripod Amphiphiles for Membrane Protein Analysis

Pil Seok Chae,*[a] Andrew C. Kruse,[d] Kamil Gotfryd,[e] Rohini R. Rana,[c] Kyung Ho Cho,[a] Søren G. F. Rasmussen,[d] Hyoung Eun Bae,[a] Richa Chandra,[f] Ulrik Gether,[e] Lan Guan,[f] Brian K. Kobilka,[d] Claus J. Loland,[e] Bernadette Byrne,*[c] and Samuel H. Gellman*[b]

chem_201301423_sm_miscellaneous_information.pdf

Supplementary Information



TRIPAO

Figure S1 Chemical structure of a TPA with N-oxide, designated TRIPAO, which is commercially available.

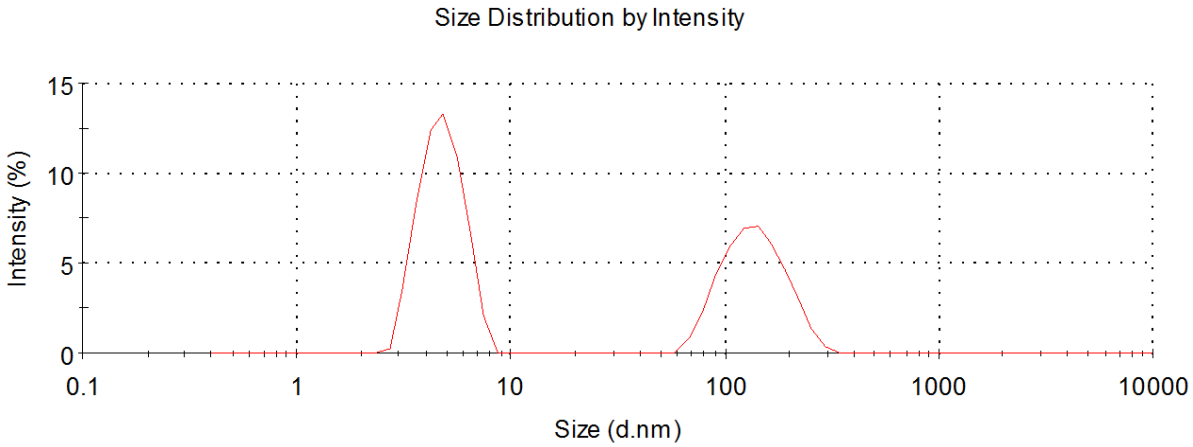


Figure S2 Representative assessment of the size distribution of micelles formed by TPA-5 at 1.0 wt % obtained by dynamic light scattering (DLS).Unlike the other TPAs and DDM, TPA-5 forms two micelle size populations with R_h of ~2.2 nm and ~75 nm. The intensity ratio of the two sets of peaks is approximately 3:2, indicating that the number ratio of the two types of micelles is > $2x10^9$:1 (light scattering intensity is proportional to R_h^6). Thus, the small aggregates with R_h of ~2.2 nm are exclusively present in the TPA-5 solution.

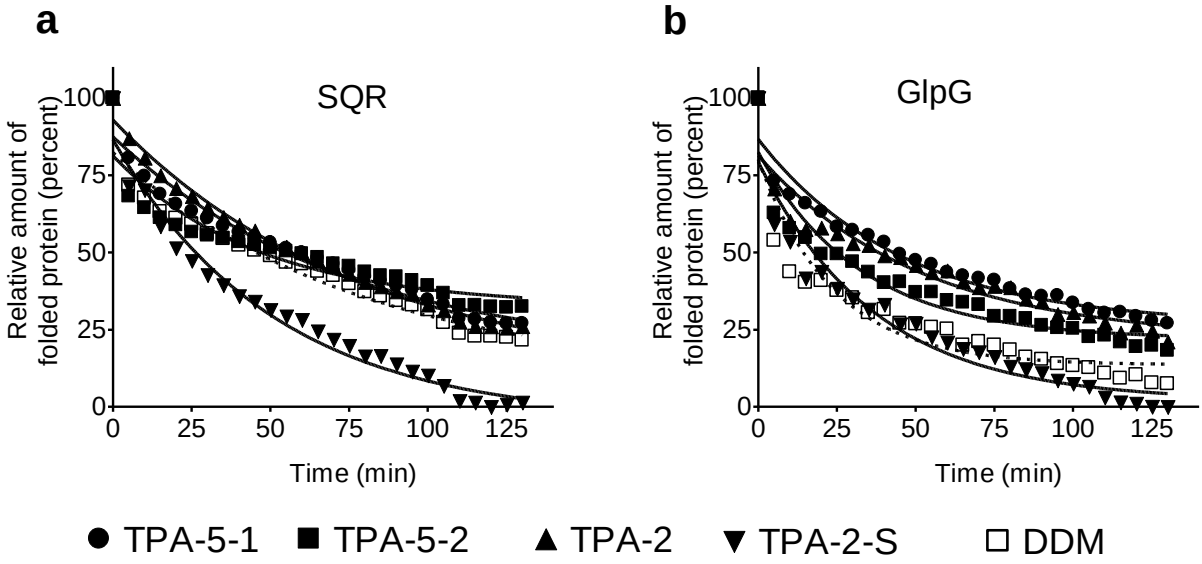


Figure S3 CPM assays for (**a**) SQR and (**b**) GlpG in tripod amphiphiles and DDM. All agents were used at CMC + 0.2 wt %. The protein unfolding in individual amphiphiles was monitored at 30 ℃ for 130 min. Results are expressed as % folding state at time 0. The coumarin moiety of CPM is internally quenched by the maleimide unit, but the coumarin becomes fluorescent following reaction with Cys side chain thiol groups exposed upon protein unfolding. The CPM assay can therefore be used to monitor the extent of protein unfolding. SQR was extracted from the membrane using 2% $C_{12}E_9$ in 20 mM potassium phosphate (pH 7.4), 0.2 M EDTA, and isolated in 20 mM Tris (pH 7.6), 0.2% decyl-β-D-maltoside (DM). GlpG was initially extracted from the native membrane with 1% DDM in PBS, 10 mM imidazole (pH 8.0), 150 mM NaCl, 10% glycerol, and isolated in 20 mM Tris (pH 7.5), 150 mM NaCl containing 0.03% DDM. The purified proteins (SQR (12 mg/ml) and GlpG (5 mg/ml) and) were diluted 1:150 in 20 mM Tris (pH 7.5), 150 mM NaCl containing CMC + 0.04 wt % amphiphile or DDM. The CPM analysis was performed over 130 min at 30°C using a microplate spectrofluorometer set at an excitation wavelength of 387 nm and an emission wavelength of 463 nm. Measurements were taken every 5 min after automatic agitation of the plate. The vertical axes in these graphs have no absolute meaning. The "Relative amount of folded protein" in each case is defined as follows: 100% corresponds to the fluorescence emission intensity at time = 0 min; 0% corresponds to the lowest value measured among the amphiphile-treated samples for each protein during the 130 min assay period. Thus, for SQR and GlpG, 0% is defined by the end-point measurement for protein solubilized with TPA-2-S. In no case can the "0%" value be interpreted as indicating that the protein is fully unfolded.

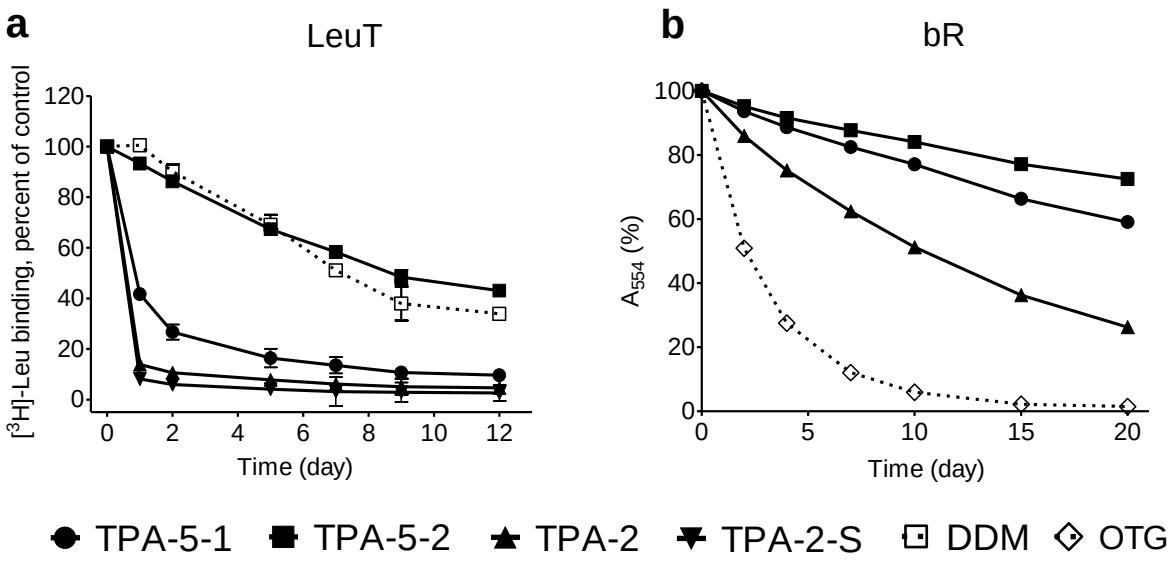


Figure S4 Time course (**a**) activity of LeuT and (**b**) stability of bR in TPAs and a conventional detergent (DDM or OTG). LeuT activity was measured employing scintillation proximity assay (SPA) based on [^{3}H]-Leu binding using protein samples stored at room temperature solubilized with the respective detergents at CMC + 0.20 wt %. bR stability was estimated via absorbance at 554 nm using a detergent mixture of 0.2 wt % OTG + 0.8 wt % TPA. Results are expressed as % activity (mean ± SEM, n = 2) or % absorbance relative to the day 0 measurements for LeuT and bR, respectively.

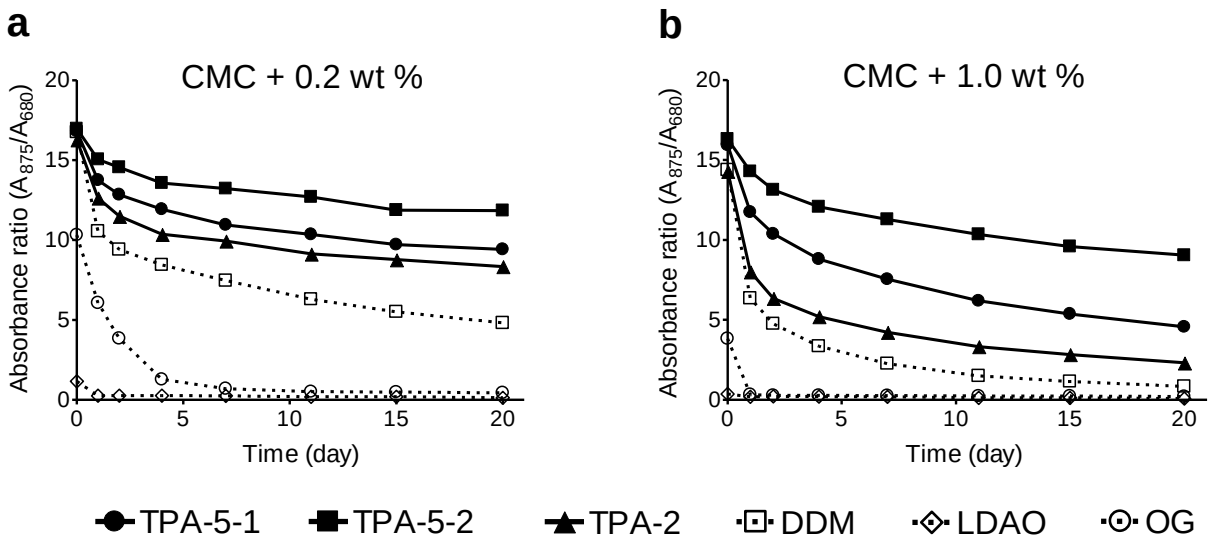


Figure S5 Time course stability of LHI-RC complex at two different detergent concentrations ((a) CMC + 0.2 wt % and (b) CMC + 1.0 wt %). Three TPAs (TPA-2, TPA-5-1 and TPA-5-2) and three conventional detergents (OG, LDAO and DDM) were evaluated with the superassembly over 20 days. The absorbance ratio (A_{875}/A_{680}) was used to assess the stability of the superassembly.

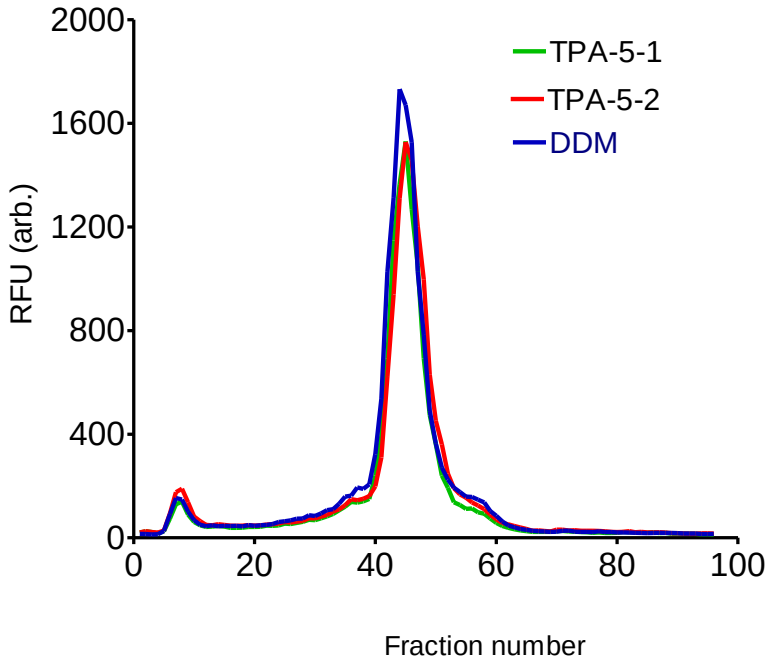
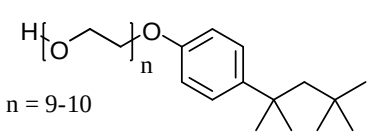
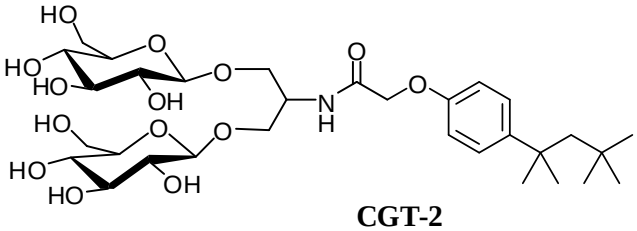


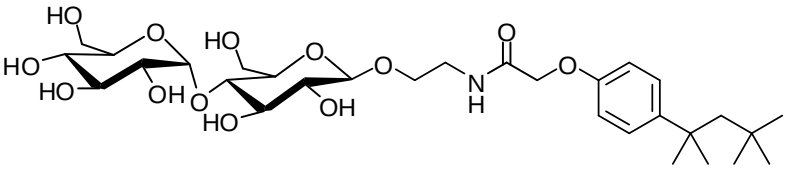
Figure S6 Characterization of CMP-Sia fusion protein bearing green fluorescent protein (GFP) at the C-terminus solubilized with TPA-5-1, TPA-5-2 and DDM. TPAs and DDM extracted ~70 % and ~80 % protein from the membrane, respectively. The fusion protein was expressed in *Saccharomyces cerevisiae* and solubilized using 1.0 % of each detergent.



Triton X-100



CGT-2



CGT-3

Figure S7 Chemical structures of Triton X-100 and its carbohydrate versions (CGT-2 and CGT-3).

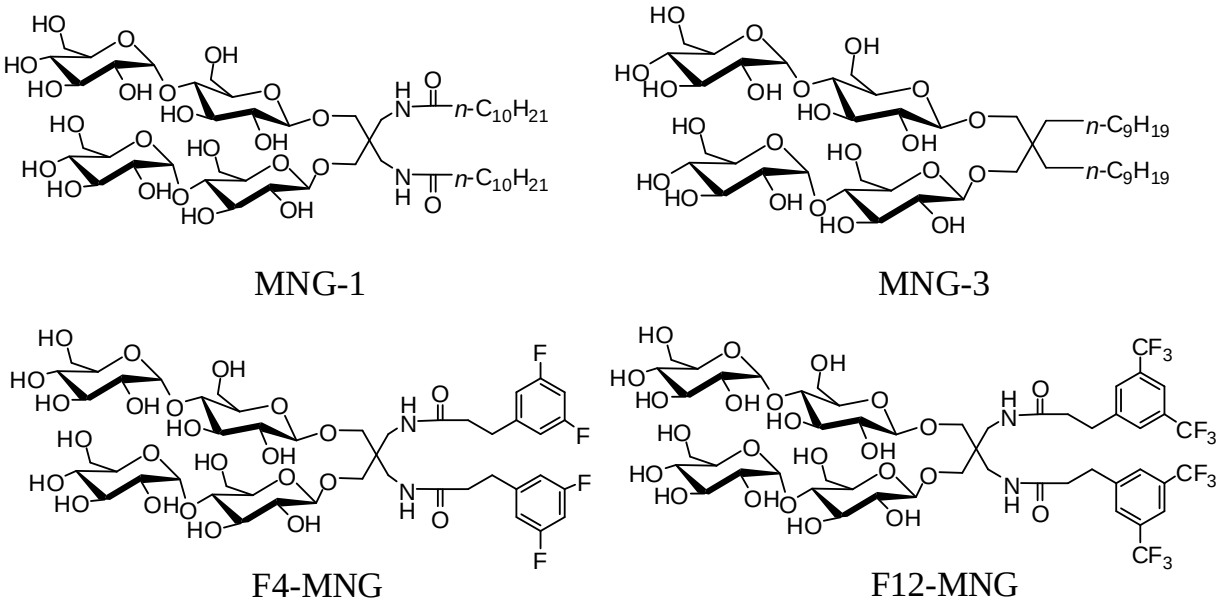


Figure S8 Chemical structures of MNG amphiphiles (MNG-1 and MNG-3) and HF-MNGs (F4-MNG and F12-MNG).

Protein stability evaluation

Membrane Solubilization and Protein Purification (CMP-Sia, GlpG and SQR)

CMP-Sia and GlpG were expressed as fusion proteins with a C-terminal GFP-His tag in *Saccharomyces cerevisiae* and *Escherichia coli* respectively. All further steps were carried out at 4 °C. Membranes containing CMP-Sia and GlpG were resuspended in PBS containing 10 mM imidazole pH 8.0, 150 mM NaCl, 10% glycerol and solubilised in 1% DDM for 1 hr with mild agitation. The supernatant containing DDM-solubilised protein was harvested after ultracentrifugation at 100,000 *g* for 45 min. The GFP-His fusions, CMP-Sia and GlpG were individually bound to Ni^{2+}-NTA resin (1 ml per 1 mg of GFP fusion) pre-equilibrated with Buffer A (PBS, 10 mM Imidazole pH 8.0, 150 mM NaCl, 10% glycerol, 0.03% DDM) using stirred mixing for 2-3 hr. The resin was washed with 10 CV of Buffer A, then 35 CV of Buffer A supplemented with 30 mM imidazole, followed by elution using 2-3 CV of Buffer A supplemented with 250 mM Imidazole. Equal amounts of His-tagged TEV protease was added to the GFP-His fusions in the eluate, and the samples dialysed overnight against Buffer B (20 mM Tris (pH 7.5), 150 mM NaCl, 0.03% DDM). Cleaved CMP-Sia and GlpG were isolated in the flowthrough fractions using reverse Ni^{2+}-NTA binding. Samples were concentrated to a 0.5 ml volume using centrifugal concentrators, and submitted to a final polishing gel filtration step using a Superdex 200 10/300 column pre-equilibrated with Buffer B. CMP-Sia and GlpG were concentrated to 6 mg/ml and 5mg/ml respectively, using molecular weight cut-off filters.

SQR was expressed in *E. coli* as an untagged construct. Membranes (~400 mg) containing SQR were resuspended in 20 mM potassium phosphate (pH 7.4), 0.2 M EDTA and solubilised in 2% $C_{12}E_9$ for 15 min. Supernatant containing detergent-solubilised protein was harvested following ultracentrifugation at 100,000*g* for 45 min, and filtered through a 0.2 μm filter. SQR was bound to pre-equilibrated Q-sepharose Fast Flow resin in an XK26/20 column (~ 24 ml). The column was washed with 2 CV of Buffer C (20 mM potassium phosphate (pH 7.4), 0.2 M EDTA, 0.05% $C_{12}E_9$), 2 CV of Buffer C supplemented with 100 mM NaCl, followed by elution using a (100-350) mM NaCl gradient. Fractions containing SQR were concentrated using an Amicon stirred cell concentrator, and filtered. The SQR was then applied onto Phoros 50 HQ resin packed into an XK16/20 column (~20 ml) pre-equilibrated with Buffer C, followed by a Sephacryl 300 26/60 pre-equilibrated with buffer D (20 mM potassium phosphate (pH 7.4), 0.05% $C_{12}E_9$). The final buffer exchange was performed on a Superdex 200 10/300 gel filtration column pre-equilibrated with 20 mM Tris (pH 7.6), 0.2% decyl-β-D-maltoside (DM). SQR was concentrated to 12 mg/ml using molecular weight cut-off filters.

Samples for CPM Assay and Gel Filtration Analysis

CPM dye (Invitrogen), stored in DMSO (Sigma), was diluted (1:100) in Buffer B supplemented with 5 mM EDTA. The TPAs or DDM were used at a concentration of CMC + 0.04 wt % or CMC + 0.2 % in 20 mM Tris (pH 7.5), 150 mM NaCl. 1 µl of the purified protein; GlpG (5 mg/ml) and SQR (12 mg/ml) was individually added to test buffers (150 µl) in Greiner 96-well plates, and left to equilibrate at RT for 5 min, before adding 3 µl diluted CPM dye.

Purification and activity assay of LeuT

LeuT activity was measured according to the reported procedure.[1] The leucine transporter (LeuT) from *Aquifex aeolicus* was expressed in *E. coli* C41(DE3) transformed with pET16b encoding LeuT WT-His_8, essentially as described.[2] Plasmid was kindly provided by E. Gouaux (Vollum Institute, Portland, Oregon, USA). Briefly, after isolation of membrane fraction followed by solubilization in 1 wt % DDM, the LeuT was bound to nickel-chelating resin (Invitrogen, USA) and eluted in 20 mM Tris-HCl (pH 8.0), 1 mM NaCl, 199 mM KCl, 0.05 % DDM in the presence of 300 mM imidazole. Subsequently, approx. 1.5 mg/ml protein stock was diluted ten-fold in the above-mentioned buffer without DDM and imidazole, but containing individual TPAs at final concentrations of CMC + 0.04 wt % or CMC + 0.2 wt %. In control samples, DDM was used at identical final concentrations. Protein samples were stored at RT, and at the indicated time points were centrifuged and the concentration was measured based on absorbance determination at 280 nm. Concomitantly, for the respective time points, [^{3}H]-Leu binding was determined employing scintillation proximity assay (SPA).[3] Briefly, SPA was carried with 5 µL from the corresponding protein samples, 20 nM [^{3}H]-Leu and 1.25 mg/ml copper chelate (His-Tag) YSi beads (both from PerkinElmer, USA) in the presence of 200 mM NaCl and tested compounds at the above-mentioned concentrations. Binding was monitored using a MicroBeta liquid scintillation counter (PerkinElmer).

Solubilization and stability assay for *R. capsulatus* superassembly

The solubilization and stability of the *R. capsulatus* superassembly were assessed according to the published protocols.[4] Briefly, we used specialized photosynthetic membranes obtained from an engineered strain of *Rhodobacter (R.) capsulatus*, U43[pUHTM86Bgl], lacking the LHII light-harvesting complex. We began solubilization of the LHI-RC superassembly by thawing, homogenizing, and incubating frozen aliquots of *R. capsulatus* membranes at 32˚C for 30 min. A subsequent 30-min incubation was performed after addition of conventional detergents (DDM) or tripod amphiphiles (TPA-2, TPA-2-S, TPA-5, TPA-5-1 or TPA-5-2) at 1.0 wt % in the solid form. The solution was then subjected to ultracentrifugation at 315,000 *g* at 4℃ for 30 min to remove membrane debris. To assess solubilization efficiency, UV-Vis spectra of the solubilized protein solutions were measured in a range of 650 ~ 950 nm.

For the stability assay, the DDM-solubilized material was transferred into a new microcentrifuge tube containing Ni^{2+}-NTA resin (Qiagen, Inc.; Valencia, CA; pre-equilibrated and stored in an equal volume of buffer containing 10 mM Tris, pH 7.8, and 100 mM NaCl). After a 1 h incubation at 4°C for binding, the resins were washed twice with 0.5 mL of binding buffer (a pH 7.8 Tris solution containing DDM at 1xCMC) and eluted three times with 0.2 mL elution buffer aliquots containing 1 M imidazole (otherwise, this buffer was identical to binding buffer; the pH of each solution was readjusted to pH = 7.8). The DDM-purified solutions were collected and diluted with 0.4 mL of the binding buffer. Then small aliquots (0.05 mL) of the DDM-purified protein solutions were mixed with 0.95 mL TPA or conventional detergent solutions at final concentrations of CMC + 0.04 wt %, CMC + 0.2 wt % or CMC + 1.0 wt %. UV-Vis spectra of these solutions were monitored at room temperature over 20 days. Protein degradation was assessed by measuring the 875/680 nm absorbance ratio.

Solubilization and stability assay of WT β_2AR

A gene encoding amino-terminally FLAG epitope tagged WT β_2AR was expressed in Sf9 cells by baculovirus, with no ligand present during culture. Cells were infected at a density of 4×10^6 cells/mL and then cultured for 48 hours prior to harvesting by centrifugation. Cells were resuspended and lysed by osmotic shock with a low ionic strength buffer (20 mM Tris pH 7.5, 1 mM EDTA). The lysed cells were frozen in aliquots containing 35 mg total protein. For extraction tests, 300 μL of solubilization buffer (20 mM HEPES pH 7.5, 100 mM NaCl) containing each amphiphile was added to one aliquot, which was then homogenized by pipet followed by grinding with a glass dounce tissue homogenizer. After a two-hour incubation at 4 °C, samples were centrifuged at maximum speed in a tabletop microcentrifuge to pellet insoluble material. Supernatant was removed and assayed for protein concentration by D_C protein assay (Bio-Rad).

The amount of functional receptor was quantified by incubation for 1 hour with 10 nM [^{3}H]dihydroalprenolol. Samples were then separated by gel filtration over G-50 resin and radioactivity was quantified by liquid scintillation. Nonspecific binding was measured in the presence of 10 μM alprenolol. Assays were performed in triplicate at the time points indicated. G-50 filtration was performed in buffer containing 20 mM HEPES pH 7.5, 100 mM NaCl, 10-fold CMC of the detergent tested. All binding assays were performed with ice cold buffers.

Bacteriorhodopsin stability

The procedure for the bR stability assay followed the reported protocol.[5] Frozen aliquots of purple membranes containing bR at 184 μM were thawed at room temperature and solubilized using 2%

octylthioglucoside (OTG) for 24 hr at 4℃ in a dark room. For this purpose, OTG (CMC = 0.28 wt %) was used at 2.0 wt % in 10 mM sodium phosphate (pH 6.9). Membrane debris was then removed from the solubilized material via ultracentrifugation at 200,000 *g* at 4℃ for 20 min. The supernatant, containing the bR protein, was transferred into individual TPAs, giving a final concentration of OTG : TPAs = 0.2 wt % : 0.8 wt % (1:4). The stability of bR in each solution was monitored by measuring absorbance at 554 nm over 20 days.

Solubilization and thermostability assay of MelB

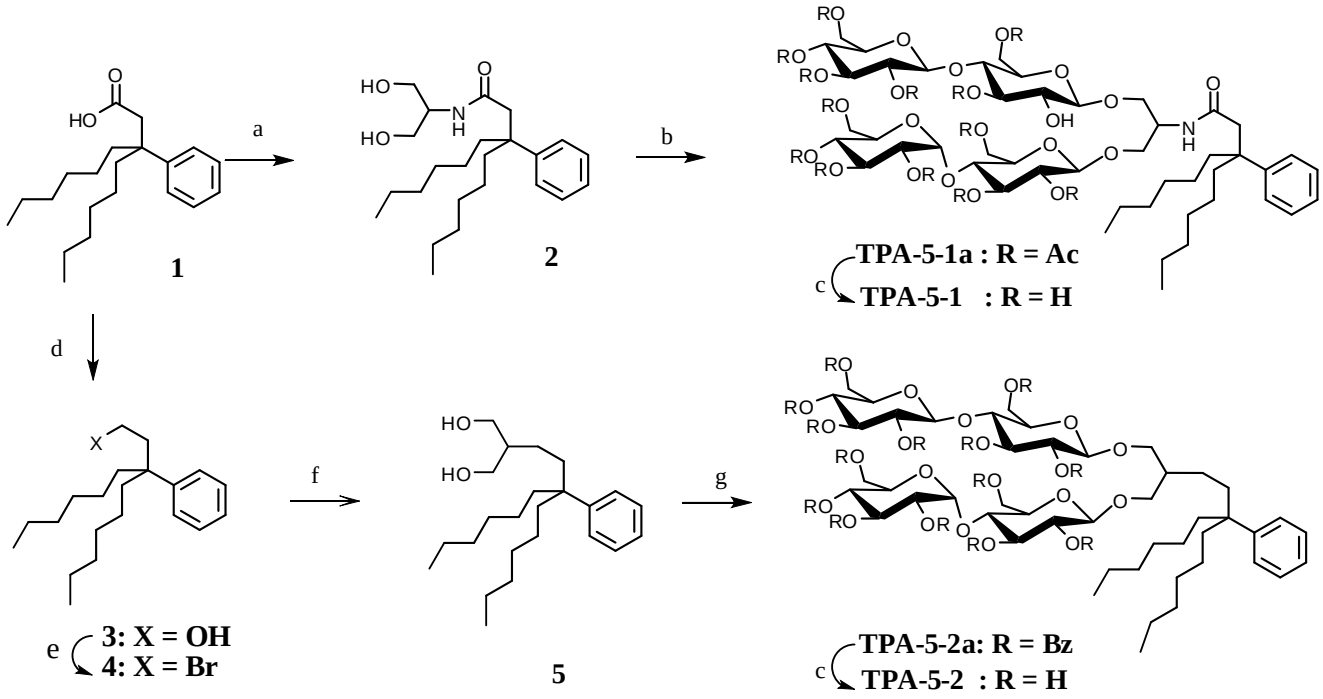
The reported protocol[2] was used to evaluate MelB stability with DDM and TPA-5-2. Wild-type MelB with a C-terminal 10-His tag was expressed in *E. coli* DW2-R cells using the expression vector pK35ΔAHB/WT MelB/CH10. Cells were harvested and resuspended in a buffer containing 20 mM Tris, pH 7.5, 200 mM NaCl and 10% glycerol. The harvested cells were lysed and resultant suspension centrifuged at 20,000 g for 15 min to remove unbroken cells. The membrane fraction was obtained via ultracentrifugation at 43,000 rpm for 3 hr in a Beckman Type *45 Ti rotor*. A protein assay was carried out with a BCA kit (Thermo Scientific, Rockford, IL). For the measurement of solubilization efficiency, membrane samples containing MelB were incubated with a solubilization buffer (20 mM Tris, 200 mM NaCl, 10% glycerol, 20 mM melibiose, pH, 7.5) and DDM or TPA-5-2 at 1.5 wt % at 0℃ for 10 min. The final protein concentration was 10 mg/mL. For the MelB thermostability, the samples were incubated for 90 min at the four different temperatures (0, 45, 55, and 65℃). After ultracentrifugation at 355,590 g in a Beckman Optima^{TM} MAX Ultracentrifuge using a TLA-100 rotor for 45 min at 4℃, 10 μg protein before and after spin for each condition was analyzed by SDS-12% PAGE and immunoblotted with Penta-His-HRP antibody (Qiagen, Germantown, MD).

CMP-Sia solubilization

CMP-Sia was expressed as a fusion protein with a C-terminal GFP in FGY217 *Saccharomyces cerevisiae* cells. Cell lysis was conducted using a cell disruptor (Constant Systems) and the protein samples were subjected to centrifugation at 15,000 *g* for 10 mins to remove unbroken cells and debris. Subsequently, the membranes were harvested by ultracentrifugation at 150,000 *g* for 45 min. The membranes were resuspended in 50 mM Tris–HCl (pH 7.6), 1 mM EDTA, 0.6 M sorbitol and the protein concentration was estimated using a BCA kit (Pierce). The membranes were incubated with DDM, TPA-5-1 and TPA-5-2 at 1.0% for 1 hr at 4°C. A fluorescence value was measured for each sample before and after ultracentrifugation at 150,000 *g* for 1 h. The solubilization efficiency was calculated via the fluorescence measurements of the soluble supernatant/the total sample.

Amphiphile Synthesis

Synthetic Scheme



(a) Serinol, EDC • HCl, HOBt, DMF, RT, 2 days; (b) 1,3-trans-peracetylated maltose (2.4 equv), $BF_3{\cdot}Et_2O$, CH_2Cl_2, RT; (c) NaOMe, MeOH, RT, 4 hr; (d) $LiAlH_4$, THF, RT, 1 day; (e) CBr_4, Ph_3P, MeCN:THF, RT, 15 hr; (f) diethylmalonate, NaH, THF, RT, 15 hr; $LiAlH_4$, THF, RT, 1 day; (g) perbenzoylated maltosylbromide, AgOTf, CH_2Cl_2, -45°C → room temperature, 3 hr.

3-hexyl-3-phenylnonanoic acid (**1**)

This acid derivative was synthesized according to the literature procedure[6] by using 2-(7-phenyltridecan-7-yl)malononitrile as a reactant. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.39-7.22 (m, 4H), 7.22-7.13 (m, 1H), 2.74 (s, 2H), 1.85-1.68 (m, 4H), 1.34-1.06 (m, 14H), 1.06-0.91 (m, 2H), 0.91-0.74 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 177.5, 146.1, 128.3, 126.4, 126.0, 43.5, 41.2, 38.9, 31.9, 30.1, 23.8, 22.8, 14.3; **HRMS (ESI)**: calcd. for $C_{21}H_{34}O_2$ $[M+Na]^+$ 341.2458, found 341.2452.

N-(1,3-dihydroxypropan-2-yl)-3-hexyl-3-phenylnonanamide (**2**)

3-hexyl-3-phenylnonanoic acid (**1**) (3.8 mmol), serinol (4.6 mmol) and 1-hydroxybenzotriazole monohydrate (HOBt) (0.61 g, 4.6 mmol) were dissolved in anhydrous DMF (30 mL). 1-(3-(Dimethylamino)propyl)-3-ethylcarbodiimide hydrochoride (EDC • HC1) (0.87 g, 4.55 mmol) was added in small portions at 0°C, and the resulting solution was stirred at room temperature for 20 h. The solution was diluted with EtOAc (100 mL) and then washed successively with 1 M aqueous $NaHCO_3$ (100 mL), 0.1 M aqueous HCl (100 mL) and brine (2 x 100 mL). The organic layer was dried with anhydrous Na_2SO_4, and solvent was removed by rotary evaporation. The crude product was precipitated with ether (100 mL), and the resulting solid was collected and dried *in vacuo* to afford

amide-containing diol (**2**) as a white solid (92%). This product was used for the next reaction without purification. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.45-7.30 (m, 4H), 7.26-7.19 (m, 1H), 5.52 (d, J = 6.5 Hz, 1H), 3.69-3.54 (m, 1H), 3.54-3.41 (m, 2H), 3.37-3.24 (m, 2H), 2.67 (br t, 2H), 2.52 (s, 2H), 1.91-1.64 (m, 4H), 1.39-1.15 (m, 14H), 1.15-0.95 (m, 2H), 0.95-0.73 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 171.8, 147.0, 128.7, 126.8, 126.3, 63.6, 52.2, 47.5, 43.7, 36.7, 31.9, 30.1, 23.6, 22.9, 14.3 ; **HRMS (ESI)**: calcd. for $C_{24}H_{41}NO_3$ $[M+Na]^+$ 414.2979, found 414.2971.

3-hexyl-3-phenylnonan-1-ol (**3**)

$LiAlH_4$ (0.44 g, 1.5 mmol) was added slowly to compound **2** (3.8 mmol) dissolved in THF (50 mL) at 0°C. The mixture was stirred at RT for 1 day, quenched with MeOH, water, a 1 N aqueous HCl solution successively at 0°C and then extracted with diethyl ether (2 x 50 mL). The combined organic layer was washed with brine and dried with anhydrous Na_2SO_4. The residue was purified by silica gel column chromatography (EtOAc/hexane) providing a desired product (**3**) as a white solid (95 %). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.41-7.23 (m, 4H), 7.23-7.11 (m, 1H), 3.46 (t, J =7.4 Hz, 2H), 1.96 (t, J = 7.6 Hz, 2H), 1.64 (t, J = 8.0 Hz, 4H), 1.37-1.15 (m, 12H), 1.15-0.96 (m, 5H), 0.96-0.72 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 147.5, 128.3, 126.5, 125.7, 59.7, 42.3, 41.1, 37.8, 32.0, 30.3, 23.5, 22.9, 14.3 ; **HRMS (ESI)**: calcd. for $C_{21}H_{36}O$ $[M+Na]^+$ 327.2659, found 327.2651.

(7-(2-bromoethyl)tridecan-7-yl)benzene (**4**)

To a solution of alcohol (**3**) (2.3 mmol) and carbon tetrabromide (3.0 mmol) in CH_2Cl_2 (100 mL) was added triphenylphosphine (Ph_3P) at 0 °C. The solution was stirred at 0°C for 1 hr and then continued the stirring at RT for 15 hr. The solvent was evaporated, and then a 1:15 mixture of CH_2Cl_2 and hexane (100 mL) was added to the residue to dissolve the crude product by not triphenylphosphine oxide. After filtration and evaporation, the residue was purified by silica gel column chromatography (eluting with EtOAc/hexane) to provide the desired product (**4**) as a white solid (92 %). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.33-7.22 (m, 4H), 7.22-7.16 (m, 1H), 3.83 (t, J = 6.8 Hz, 2H), 2.25 (t, = 7.2 Hz, 2H), 1.63 (t, = 7.4 Hz, 2H), 1.38-1.15 (m, 12H), 1.15-0.95 (m, 4H), 0.95-0.72 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 146.3, 128.5, 126.0, 111.0, 44.7, 42.4, 37.3, 31.9, 30.2, 29.4, 23.5, 22.9, 14.3; **HRMS (ESI)**: calcd. for $C_{21}H_{35}Br$ $[M]^+$ 366.1917, found 366.1929.

2-(3-hexyl-3-phenylnonyl)propane-1,3-diol (**5**)

To a solution of bromide **4** (2.1 mmol) and diethyl malonate (10.4 mmol) in a 1:1 mixture of THF and DMF (80 mL) was added K_2CO_3 (10.5 mmol). The mixture was heated at 90 °C for 15 hr, and then water (100 mL) was added at 0°C. The resulting solution was extracted with diethyl ether (2 x 100 mL). The combined organic layer was washed with brine and dried with anhydrous Na_2SO_4. After

removal of solvent, the crude product was used for the next reaction without purification. The crude product was dissolved in THF (50 mL), and $LiAlH_4$ (14.0 mmol) was added slowly to the solution at 0°C. The mixture was stirred at RT for 1 day, and then excess hydride was quenched by addition of MeOH, water and 1 N aqueous HCl successively at 0°C. The resulting solution was extracted with diethyl ether (2 x 50 mL). The combined organic layer was washed with brine and dried with anhydrous Na_2SO_4. After removal of solvent, the residue was purified by silica gel column chromatography (eluting with EtOAc/hexane) to provide diol (**6**) as a white solid (93% (two steps)). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.32-7.24 (m, 4H), 7.20-7.11 (m, 1H), 3.74 (dd, J = 10.6, 3.8 Hz, 2H), 3.57 (dd, J = 10.6, 7.7 Hz, 2H), 2.11 (s, 2H), 1.72-1.54 (m, 8H), 1.32-1.14 (m, 12H), 1.11-0.89 (m, 6H), 0.89-0.78 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 147.8, 128.2, 126.7, 125.5, 66.8, 43.3, 42.9, 37.3, 35.8, 32.0, 30.3, 23.6, 22.9, 21.7, 14.3 ; **HRMS (ESI)**: calcd. for $C_{24}H_{42}O_2$ $[M+Na]^+$ 385.3078, found 385.3063.

TPA-5-1a was synthesized from diol **2** according to according to the literature method[7] with slight modification. $BF_3{\cdot}Et_2O$ (3 equiv with respect to protected maltose) was added to the 1,2-trans peracetylated maltose (2.4 equiv) and the alcohol derivatives (1.0 equiv) in dry CH_2Cl_2 under a nitrogen atmosphere at room temperature. The reaction was monitored by TLC. When the reaction did not progress further (usually 48 h), the mixture was diluted with CH_2Cl_2, and washed with saturated aqueous $NaHCO_3$ and then water. The organic layer was dried with Na_2SO_4, and concentrated by rotary evaporation. The residue was purified by silica gel column chromatography (EtOAc/ hexane 1:1) providing desired product as a glassy solid (73%). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.46-7.26 (m, 4H), 7.26-7.16 (m, 1H), 5.46-5.31 (m, 4H), 5.27-5.16 (m, 2H), 5.16-5.00 (m, 3H), 4.92-4.81 (m, 2H), 4.78-4.67 (m, 2H), 4.52-4.41 (m, 2H), 4.41-4.30 (m, 2H), 4.30-4.16 (m, 4H), 4.16-3.90 (m, 7H), 3.74-3.59 (m, 2H), 3.58-3.35 (m, 3H), 3.12-3.03 (m, 1H), 2.46 (s, 2H), 2.20-1.94 (m, 42H), 1.86-1.64 (m, 4H), 1.36-0.95 (m, 16H), 0.95-0.77 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 170.7, 170. 6, 170.5, 170.2, 170.0, 169.6, 169.5, 146.6, 128.5, 126.5, 126.1, 100.8, 100.6, 95.7, 75.4, 75.3, 72.9, 72.8, 72.3, 72.1, 70.1, 69.5, 69.4, 68.6, 68.2, 68.1, 67.6, 62.9, 61.6, 47.8, 46.3, 43.4, 37.3, 36.8, 31.9, 31.8, 30.1, 30.0, 23.6, 22.8, 21.0, 20.9, 20.8, 20.7, 14.2 ; **MS (MALDI-TOF)**: calcd. for $C_{76}H_{109}O_{37}$ $[M+Na]^+$ 1650.7, found 1650.9.

TPA-5-2a was synthesized from diol **5** according to a reported method[8] with slight modifications. A mixture of the alcohol to be glycosylated, AgOTf (2.4 equiv.), and 2,4,6-collidine (2.0 equiv.) in anhydrous CH_2Cl_2 (40 mL) was stirred at -45°C. A solution of perbenzoylated maltosylbromide (2.4 equiv.) 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 allowed to warm to 0 °C and stirred for 1.5 h. After completion of the reaction (as detected by TLC), pyridine was added to the reaction mixture,

which was then diluted with CH_2Cl_2 (40 mL) and filtered through celite. The filtrate was washed successively with 1 M aqueous $Na_2S_2O_3$ solution (40 mL), 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 solvent was removed by rotary evaporation. The residue was purified by silica gel column chromatography (eluting with EtOAc/hexane) to provide the desired product as a glassy solid (92%). **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.09-7.99 (m, 5H), 7.99-7.91 (m, 10H), 7.91-7.82 (m, 4H), 7.82-7.76 (m, 4H), 7.76-7.63 (m, 5H), 7.63-7.14 (m, 44H), 7.14-7.02 (m, 1H), 6.13 (td, *J* = 10.2, 5.1 Hz, 2H), 5.73-5.58 (m, 4H), 5.35 (td, *J* = 9.8, 4.4 Hz, 2H), 5.23-5.11 (m, 2H), 5.11-5.00 (m, 2H), 4.76-4.48 (m, 4H), 4.48-4.06 (m, 8H), 3.65 (d, *J* = 8.1 Hz, 1H), 3.54-3.44 (m, 1H), 3.24 (dd, *J* = 7.8, 4.3 Hz, 2H), 3.09-2.92 (m, 3H), 2.68 (t, *J* = 9.4 Hz, 1H), 1.61-1.30 (m, 6H), 1.30-1.06 (m, 14H), 1.06-0.61 (m, 14H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 166.3, 166.2, 166.0, 165.7, 165.3, 165.2, 165.1, 164.7, 147.9, 134.0, 133.7, 133.6, 133.4, 133.3, 133.2, 133.1, 133.0, 129.9, 129.8, 129.7, 129.6, 129.5, 129.4, 129.3, 129.2, 129.1, 129.0, 128.9, 128.8, 128.6, 128.5, 128.3, 128.0, 126.7, 125.3, 101.2, 101.0, 96.0, 95.7, 74.8, 74.7, 72.3, 72.1, 71.4, 70.0, 69.3, 69.2, 63.5, 62.7, 43.0, 38.9, 38.3, 36.9, 34.4, 32.1, 32.0, 30.4, 30.3, 23.5, 23.4, 23.0, 22.9, 21.2, 14.4, 14.3; **MS (MALDI-TOF)**: calcd. for $C_{146}H_{138}O_{36}Na$ $[M+Na]^+$ 2489.9, found 2490.0

General Procedure for de-*O*-aceylations or de-*O*-benzoylations[8]

O-Benzoylated compounds were dissolved in MeOH and 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 stirred for 6 h at RT, and then neutralized with Amberlite IR-120 resin (H^+ form). The resin was removed by filtration and washed with MeOH, and the solvent was removed from the combined filtrate *in vacuo*. The residue was purified by silica gel column chromatography (eluting with MeOH/CH_2Cl_2). Further purification was achieved by recrystallization from CH_2Cl_2/MeOH/diethyl ether, to provide the fully de-*O*-benzoylated product as a white solid.

TPA-5-1 was synthesized from **TPA-5-1a** and was obtained in a nearly quantitative yield according to the general procedure for de-*O*-benzoylation. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.43-7.30 (m, 4H), 7.30-7.15 (m, 1H), 5.19 (d, *J* = 3.5 Hz, 2H), 4.27 (t, *J* = 8.3 Hz, 2H), 4.16-4.03 (m, 1H), 4.00-3.77 (m, 6H), 3.77-3.59 (m, 10H), 3.59-3.40 (m, 4H), 3.40-3.18 (m, 6H), 2.62 (s, 2H), 1.99-1.76 (m, 4H), 1.41-1.20 (m, 12H), 1.20-1.06 (m, 4H), 1.00-0.81 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 173.9, 147.9, 129.4, 127.8, 127.0, 104.7, 103.1, 81.4, 77.9, 76.8, 75.2, 74.9, 74.8, 74.7, 74.3, 71.6, 62.9, 62.4, 44.8, 39.4, 38.8, 33.1, 31.2, 24.8, 23.8, 14.6; **HRMS (ESI)**: calcd. for $C_{48}H_{81}O_{23}$ $[M+Na]^+$ 1062.5092, found 1062.5133.

TPA-5-2 was synthesized from **TPA-5-2a** and was obtained in a nearly quantitative yield according to the general procedure for de-*O*-benzoylation. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 7.39-7.26 (m, 4H),

7.19-7.12 (m, 1H), 5.18 (d, *J* = 3.5 Hz, 2H), 4.32 (d, *J* = 7.7 Hz, 2H), 3.98-3.78 (m, 8H), 3.78-3.58 (m, 10H), 3.58-3.43 (m, 4H), 3.43-3.20 (m, 6H), 1.90- 1.74 (m, 2H), 1.74-1.65 (m, 5H), 1.38-1.19 (m, 12H), 1.19-0.99 (m, 6H), 0.95-0.84 (m, 6H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 141.2, 129.1, 127.8, 126.4, 104.8, 104.9, 103.1, 81.7, 81.6, 78.0, 76.7, 75.2, 74.9, 74.3, 71.6, 71.4, 70.6, 62.9, 62.5, 62.4, 44.3, 41.5, 38.7, 38.6, 33.1, 31.3, 24.6, 23.8, 23.6, 14.6; **HRMS (ESI)**: calcd. for $C_{48}H_{82}O_{22}$ $[M+Na]^+$ 1033.5190, found 1033.5211.

References

[1] M. Quick, J. A. Javitch, *Proc. Natl. Acad. Sci. U. S. A.* **2007**, *104*, 3603-3608.

[2] P. S. Chae, *et al*., *Nat. Methods* **2010**, *7*, 1003-1008.

[3] P. S. Chae, et al., *Chem.-Eur. J.* **2012**, *18*, 9485-9490

[4] P. S. Chae, *et al*., *J. Am. Chem. Soc.* **2010**, *132*, 16750-16752.

[5] P. Bazzacco, K. S. Sharma, G. Durand, F. Giusti, C. Ebel, J.-L. Popot, B. Pucci, *Biomacromolecules* **2009**, *10*, 3317-3326.

[6] S. M. Yu, D. T. McQuade, M. A. Quinn, C. P. R. Hackenberger, M. P. Krebs, A. S. Polans, S. H. Gellman, *Protein Sci*., **2000**, *9*, 2518–2527.

[7] L. A. Salvador, M. Elofsson, J. Kihlberg, *Tetrahdron* **1995**, *51*, 5643-5656

[8] P. R. Ashton, S. E. Boyd, C. L. Brown, N. Jayaraman, S. A. Nepogodiev, J. F. Stoddart, *Chem.-Eur. J*. **1996**, *2*, 1115-1128.