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Synthesis and Properties of Dodecyl Trehaloside Detergents for Membrane Protein Studies

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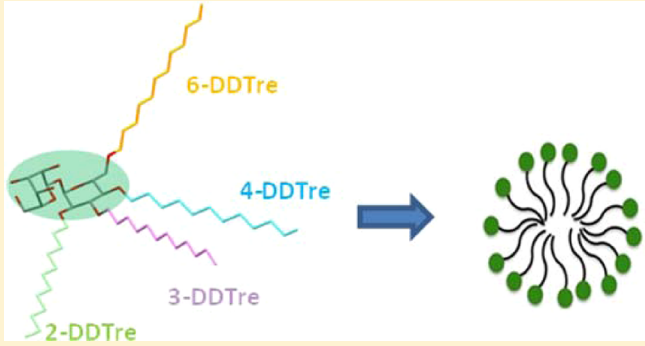
Supporting Information

ABSTRACT: Sugar-based detergents, mostly derived from maltose or glucose, prevail in the extraction, solubilization, stabilization, and crystallization of membrane proteins. Inspired by the broad use of trehalose for protecting biological macromolecules and lipid bilayer structures, we synthesized new trehaloside detergents for potential applications in membrane protein research. We devised an efficient synthesis of four dodecyl trehalosides, each with the 12-carbon alkyl chain attached to different hydroxyl groups of trehalose, thus presenting a structurally diverse but related family of detergents. The detergent physical properties, including solubility, hydrophobicity, critical micelle concentration (CMC), and size of micelles, were evaluated and compared with the most popular maltoside analogue, β-D-dodecyl maltoside (DDM), which varied from each other due to distinct molecular geometries and possible polar group interactions in resulting micelles. Crystals of 2-dodecyl trehaloside (2-DDTre) were also obtained in methanol, and the crystal packing revealed multiple H-bonded interactions among adjacent trehalose groups. The few trehaloside detergents were tested for the solubilization and stabilization of the nociceptin/orphanin FQ peptide receptor (ORL1) and MsbA, which belong to the G-protein coupled receptor (GPCR) and ATP-binding cassette transporter families, respectively. Our results demonstrated the utility of trehaloside detergents as membrane protein solubilization reagents with the optimal detergents being protein dependent. Continuing development and investigations of trehaloside detergents are attractive, given their interesting and unique chemical−physical properties and potential interactions with membrane lipids.



1. INTRODUCTION

Trehalose, also known as mycose, tremalose, or α,α-trehalose, is an atypical, nonreducing disaccharide composed of two D-glucoses with an α,α-1,1′-glycosidic linkage. The nonreducing glycosidic bond of trehalose confers high chemical stability and is resistant to hydrolysis under mildly acidic conditions and elevated temperature. Trehalose is widely used in life sciences as an additive reagent for the protection of protein molecules.[1] The exceptional chemical stability of its glycosidic bond prevents the Maillard reaction with protein side chains. The stabilization effect of trehalose may also be linked to the noncovalent interaction with solvent molecules, protein surfaces, and its peculiar structural and physical properties, such as relatively low conformational flexibility and high viscosity.[1−4]

It is also widely acknowledged that high concentrations of trehalose help maintain the membrane structural and functional integrity during the dehydration and rehydration process, an effect unsurmounted by other sugars.[5−7] In addition, trehalose is abundantly found in a variety of anhydrobiotic organisms where the cumulated amount can be up to 20% of the dry weight. The protection mechanism of trehalose is partly explained by its association with cellular phospholipid head groups by the displacement water molecules, possibly via H-bonds between the trehalose hydroxyl groups and the lipid phosphate groups, which exert enhanced stability of lipid bilayer structures.[5,8,9] Accordingly, trehalose can inhibit the liposome fusion and lipid phase transitions during dehydration. In addition, trehalose-derived lipids are naturally present in some cell membranes. For example, 6,6′-diacyl trehalose lipids are the most abundant extractable lipids from the outer cell wall of *Mycobacteria tuberculosis*.[10,11] Similar diacyl trehalose lipids were recently found specific to dauer larva in *Caenorhabditis elegans* in response to harsh environmental conditions.[12] With the alkyl chains of trehalose lipids anchored into a lipid bilayer, the trehalose moiety was assumed to have a more effective interaction with phospholipids, thereby making the bilayer highly stable and impermeable and resistant to desiccation.[13,14]

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Given the protective function of trehalose on proteins and lipid bilayers, we speculate that trehalose-derived detergents are of special utility in membrane protein studies. The use of detergents is indispensable in the preparation of membrane protein samples prior to biochemical and biophysical characterization, although the stability of these proteins is often a serious concern.[15] Of note, sugar-based detergents, mostly derived from the maltose headgroup, prevail in the solubilization, purification, stabilization, and structural studies of membrane proteins, in particular for challenging eukaryotic targets.[16] The prevalence of noncharged maltoside detergents can be attributed to their relatively mild, nondenaturing properties and their increasing success in highly demanding crystallization efforts. It is worthwhile to emphasize that the selection of a detergent molecule, using not only the chain length but also the polar group, has a profound effect on the stability and crystallization of a protein target. Nonetheless, how the polar functionality of a detergent molecule affects the crystallization outcome is poorly understood. Part of the reason is that there is only a limited selection of useful detergent molecules, among which a majority are maltose derived. From this viewpoint, development of detergents with new polar functionalities, such as trehalose herein, is of particular interest for membrane protein research. But to the best of our knowledge, no effort of synthesizing and testing trehaloside detergents has been exercised in the field.

In this study, we describe our efforts toward the synthesis and property evaluation of new trehaloside detergents along with our long-term goal of developing new chemical tools for the study of membrane proteins. We also report the crystal structure of one trehaloside detergent (2-DDTre) which, compared to the free trehalose crystal structures, suggests that the trehaloside polar head is relatively inflexible in conformation. The crystal packing also reveals interesting features of polar and nonpolar interactions, which aid in our understanding of the detergent physical properties in the solution state. Finally, we evaluate the new trehaloside detergents for the solubilization and stabilization of two membrane protein systems, thereby providing clues for their future applications.

2. MATERIALS AND METHODS

2.1. General Method. All organic reactions were carried out under anhydrous conditions and an argon atmosphere, unless otherwise noted. NMR spectra were recorded using Bruker DRX-500, or Varian Inova-400 instruments, which were calibrated using residual undeuterated solvent as an internal reference. Flash column chromatography was performed using 60 Å silica gel (Acros) as a stationary phase. Thin-layer chromatography (TLC) was performed using glass-backed silica gel 60_{F254} (Merck, 250 μm thickness). The single-crystal X-ray diffraction studies were carried out on a Bruker D8 Smart 6000 CCD diffractometer equipped with Cu K_α radiation (Bruker FR-591 Rotating Anode Generator/Montels Optics λ = 1.5478). *n*-Dodecyl β-D-maltoside (DDM) was purchased from Anatrace.

2.2. Synthesis of 2-DDTre and 3-DDTre. A solution of 4,6:4′,6′-di-*O*-benzylidene trehalose (**1**)[17] (6.0 g, 11.5 mmol) in DMF (100 mL) was treated with NaH (60% dispersion, 1.0 g, 25.0 mmol) at room temperature for 2 h. To this suspension was added dropwise *n*-dodecyl bromide (3.0 mL, 12.5 mmol) over a period of 30 min. The resulting mixture was stirred for 48 h at room temperature. Then the reaction was quenched by careful addition of 1% HCl solution and extracted with ethyl acetate twice. The combined organic phases were washed sequentially with saturated $NaHCO_3$ and NaCl solutions. The organic layer was separated, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel chromatography (eluent: hexanes/EtOAc = 3:1 and 100% acetone), which gave a nonseparable mixture of 2- and 3-dodecylated products (**2** + **3**: 3.2 g, 40%). Unreacted starting material (**1**: 1.0 g, 18%) was also recovered.

The above mixture of **2** and **3** was dissolved in methanol and treated with *p*-toluene sulfonic acid (500 mg, 2.6 mmol) with stirring for 5 h at room temperature. Upon completion by TLC examination, methanol was evaporated in vacuo. The crude product was mixed with dry silica gel powder and further dried under vacuum. The resulting preabsorbed silica gel was loaded to silica column (gradient eluent: DCM/MeOH = 20:1 to 4:1), by which 2-DDTre (1.0 g, 42%) and 3-DDTre (1.1 g, 46%) was purified individually. 2-DDTre: ^{1}H NMR (500 MHz, CD_3OD) δ 5.28 (d, J = 3.4 Hz, 1H), 5.11 (d, J = 3.7 Hz, 1H), 3.89 − 3.65 (m, 9H), 3.58 − 3.53 (m, 1H), 3.48 (dd, J = 9.7, 3.7 Hz, 1H), 3.39 − 3.33 (m, 2H), 3.22 (dd, J = 9.7, 3.7 Hz, 1H), 1.70 − 1.56 (m, 2H), 1.29 (s, 18H), 0.90 (t, J = 6.9 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 95.8, 93.4, 81.4, 74.8, 74.0, 73.9, 73.8, 73.4, 72.6, 72.0, 71.7, 62.7, 62.6, 33.2, 31.3, 31.0, 30.9, 30.6, 27.3, 23.9, 14.6. ESI-MS calcd for $C_{24}H_{46}O_{11}Na$ ($[M + Na]^+$) 533.3, found 533.3. 3-DDTre: ^{1}H NMR (500 MHz, CD_3OD) δ 5.12 (d, J = 3.6 Hz, 1H), 5.10 (d, J = 3.5 Hz, 1H), 3.87 − 3.75 (m, 7H), 3.67 (m, 2H), 3.60 (t, J = 9.2 Hz, 1H), 3.55 − 3.47 (m, 2H), 3.39 − 3.33 (m, 2H), 1.70 − 1.56 (m, 2H), 1.29 (s, 18H), 0.90 (t, J = 6.8 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 95.1, 94.9, 83.2, 74.6, 74.5, 74.0, 73.9, 73.3, 73.2, 72.1, 71.7, 62.74, 62.69, 33.2, 31.6, 30.93, 30.91, 30.89, 30.87, 30.6, 27.3, 23.9, 14.6. ESI-MS calcd for $C_{24}H_{46}O_{11}Na$ ($[M + Na]^+$) 533.3, found 533.3.

2.3. Synthesis of 4-DDTre and 6-DDTre. 2,2′,3,3′-Tetra-*O*-benzyltrehalose (**4**)[18] was prepared in 80% yield from di-*O*-benzylidene trehalose (**1**) by tetra-*O*-benzylation and subsequent benzylidene deprotection. A solution of the tetrabenzyltrehalose **4** (2.1 g, 3.0 mmol) in DMF (18 mL) was treated with NaH (60% dispersion, 300 mg, 7.5 mmol) at room temperature for 2 h. To this suspension was added dropwise *n*-dodecyl bromide (0.6 mL, 2.4 mmol) over a period of 30 min. The resulting mixture was stirred for 14 h at room temperature. The reaction was quenched by 1% HCl aqueous solution and extracted with ethyl acetate twice. The combined organic phases were washed with saturated $NaHCO_3$ and NaCl solution, dried over Na_2SO_4, filtered, and concentrated in vacuo. The residue was purified by silica gel chromatography (eluent: hexanes/acetone = 8:1 to 2:1) to separate 4- and 6-dodecylated products (**5**: 520 mg, 20%; **6**: 653 mg, 25%). Unreacted starting material **4** (614 mg, 29%) was recovered at the same time. The NMR characterization data for **5** and **6** are reported in the Supporting Information (SI). Hydrogenation of **5** and **6** in the presence of 10% Pd/C catalyst was conducted in methanol, affording the final products 4-DDTre and 6-DDTre, respectively. 4-DDTre: ^{1}H NMR (500 MHz, CD_3OD) δ 5.11 (d, J = 3.8 Hz, 1H), 5.09 (d, J = 3.7 Hz, 1H), 3.90 − 3.84 (m, 2H), 3.84 − 3.74 (m, 5H), 3.69 − 3.65 (m, 2H), 3.59 − 3.56 (m, 1H), 3.49 − 3.45 (m, 2H), 3.35 − 3.33 (m, 1H), 3.20 (dd, J = 9.9, 9.0 Hz, 1H), 1.63 − 1.52 (m, 2H), 1.37 − 1.25 (m, 18H), 0.90 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 95.1, 95.0, 79.8, 74.7, 74.5, 74.1, 73.9, 73.4, 73.2, 73.0, 72.0, 62.7, 62.3, 33.2, 31.5, 30.9, 30.9, 30.9, 30.8, 30.6, 27.3, 23.8, 14.6. ESI-MS calcd for $C_{24}H_{46}O_{11}Na$ ($[M + Na]^+$) 533.3, found 533.3. 6-DDTre: ^{1}H NMR (500 MHz, CD_3OD) δ 5.11 (d, J = 4.0 Hz, 1H), 5.10 (d, J = 4.0 Hz, 1H), 3.93 (ddd, J = 10.0, 5.1, 2.1 Hz, 1H), 3.85 − 3.79 (m, 4H), 3.72 − 3.65 (m, 2H), 3.62 (dd, J = 11.1, 5.2 Hz, 1H), 3.57 − 3.43 (m, 4H),

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3.36 − 3.33 (m, 2H), 1.61 − 1.53 (m, 2H), 1.37 − 1.25 (m, 18H), 0.90 (t, J = 7.0 Hz, 3H). ^{13}C NMR (125 MHz, CD_3OD) δ 95.2, 95.1, 74.7, 74.6, 73.9, 73.3, 73.2, 72.9, 72.7, 72.2, 72.0, 71.3, 62.7, 33.2, 30.93, 30.89, 30.82, 30.79, 30.6, 27.3, 23.9, 14.6. ESI-MS calcd for $C_{24}H_{46}O_{11}Na$ ($[M + Na]^+$) 533.3, found 533.3.

2.4. Evaluation of Detergent Hydrophobicity by Reverse-Phase HPLC. HPLC was performed on a Shimadzu instrument equipped with both a UV detector and an evaporative light-scattering detector (ELSD). The detergent sample was analyzed using a C18 column (Phenomenex, Cat. 309020-1, Gemini 5 μ, 150 mm × 4.60 mm) and a mobile phase of 50% CH_3CN/50% H_2O at a flow rate of 1.0 mL/min. Assignment of each detergent peak was confirmed by multiple single and coinjections. The HPLC retention factor (k'), used for ranking the hydrophobicity of each detergent, was calculated according to the equation: $k' = (t_r - t_0)/t_0$, where t_0 = retention time of the solvent front and t_r = retention time of the detergent molecule.[19]

2.5. Measurement of Critical Micelle Concentration (CMC). The CMC value of each detergent was determined by monitoring the fluorescence (λ_{ex} = 388 nm, λ_{em} = 477 nm) of the ammonium salt of 8-anilino-1-naphtalenesulfonic acid (ANS), which undergoes an increase in the fluorescence emission when incorporated into the hydrophobic micellar environment.[20] Solutions containing 10 μM ANS and different concentrations of detergents were measured at room temperature on a Cary Eclipse fluorescence spectrophotometer (Varian). All measurements were performed in triplicate using separately prepared solutions. The CMC was defined as the inflection point in the plot of fluorescence intensity versus detergent concentration.

2.6. Dynamic Light Scattering (DLS) Measurements. The hydrodynamic radius (R_h) of detergent micelles was determined at 25 °C using a DynaPro Plate Reader (Wyatt Technology Corporation, CA). Each detergent being tested was solubilized in deionized (di) water at 0.2% (w/v), a concentration well above the CMCs. 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 viscosity value of pure water was used for all analyses. Ten acquisitions were collected for each sample, and averages for triplicate experiments were used for the analysis.

2.7. ORL1 Purification. The wild-type human ORL1 gene (encoded by *OPRL1*; UniProt accession P41146) was synthesized by DNA2.0, and then cloned into a modified pFastBac1 vector (Invitrogen) containing an expression cassette with a hemagglutinin signal sequence followed by a Flag tag at the N terminus, and a PreScission protease site followed by a 10 × His tag at the C terminus. Thirty-one amino acids were deleted from the C-terminus (residues 341−370), and 43 residues of the N-terminus (residues 1−43) of ORL1 were replaced with the thermostabilized apocytochrome b_{562}RIL from *Escherichia coli* (M7W, H102I, and K106L) (BRIL) protein. BRIL-ORL1 was expressed in *Spodoptera frugiperda* (*Sf*9) insect cells and purified as described previously.[21] Briefly, purified cell membranes were incubated with a stabilizing antagonist (5 μM C-24: 1-benzyl-*N*-{3-[spiroisobenzofuran-1(3*H*),40-piperidin-1-yl]propyl}-pyrrolidine-2-carboxamide),[22] 50 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM KCl, and 5% glycerol (v/v), and incubated at 4 °C for 1 h. Iodoacetamide (Sigma) was then added to the membranes at a final concentration of 1 mg/mL for another 15 min before solubilization with 0.5% (w/v) each detergent being tested for 3 h at 4 °C. The supernatant was isolated by centrifugation at 160,000*g* for 45 min, supplemented with 25 mM imidazole (pH 7.5) and incubated with TALON IMAC resin (Clontech) overnight at 4 °C. After binding, the resin was divided and washed with 20 column volumes of wash buffer 1 [50 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM KCl, 10 mM $MgCl_2$, 1 mM ATP, 10% glycerol (v/v), 25 mM imidazole] containing 5 μM C-24 and 5 × CMC of each detergent being tested, and 25 column volumes of wash buffer 2 (same as wash buffer 1, but without ATP and $MgCl_2$), before protein elution with 4 column volumes of elution buffer [50 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM KCl, 10% glycerol (v/v), 250 mM imidazole] containing C-24 and 5 × CMC of each detergent being tested. The eluted protein in each detergent was assayed for purity by SDS gel electrophoresis.

2.8. Thermal Stability Assay. Thermal stability data were collected using a modified procedure incorporating a thiol-reactive fluorophore, *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM), which undergoes an increase in the fluorescence emission upon binding with cysteine residues.[23] Reactions contained 5 μM CPM and ∼5 μg purified ORL1 in a buffer (elution buffer, but without imidazole) containing 50 mM HEPES (pH 7.5), 500 mM NaCl, 20 mM KCl, 10% glycerol (v/v), 5 μM C-24, and 5 × CMC of each detergent being tested. Fluorescence emission was measured on a Cary Eclipse fluorescence spectrophotometer (λ_{ex} = 387 nm; λ_{em} = 463 nm) from 20 − 90 °C with 1 °C intervals and a ramp rate of 2 °C/min, and the background fluorescence of buffer in the absence of protein was subtracted. Midpoints of the thermal transitions (T_m) were obtained using a least-squares nonlinear regression analysis (GraphPad Prism) of fluorescence signal versus T plots according to the equation described previously.[21]

2.9. MsbA Purification. The recombinant MsbA construct, originated from *E. coli*, was kindly provided by Dr. G. Chang. EcMsbA was prepared and purified as described previously.[24] Briefly, the bacterial cells overexpressing EcMsbA were directly solubilized in 1% detergent solution containing 20 mM Tris, 20 mM NaCl (pH 8.0), 10% glycerol, 0.1 mg/mL of DNase I, and proteinase inhibitor cocktail. The supernatant after centrifugation at 38,000*g* for 45 min was subject to Ni-affinity column for purification. Three × CMC of each detergent being tested was included in the wash and elution steps. MsbA was further purified by a desalting column using buffer containing 3 × CMC of each detergent, 20 mM Tris (pH 7.5), and 20 mM NaCl. The protein purity was assessed by SDS gel electrophoresis.

2.10. ATPase Activity. ATPase activity was measured using an ATP-regenerating system described by Vogel and Steinhart,[25] and modified by Urbatsch et al.[26] Briefly, 1−2 μg of MsbA purified in each detergent being tested was added to 100 μL of linked enzyme (LE) buffer at 37 °C containing 10 mM ATP, 12 mM MgCl2, 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 340 nm using a DXT880 multiplate spectrofluorimeter (Beckman-Coulter). ATPase activity was calculated using the following equation: $\Delta OD^*\varepsilon/([protein]^*time)$, where ΔOD is the change in optical density and ε is the extinction coefficient.

3. RESULTS AND DISCUSSION

3.1. Synthesis of Trehaloside Detergents. The industrial production of trehalose at significantly lowered cost has been achieved in recent years,[27] making possible the medium to large-scale synthesis of trehaloside detergents at reasonable cost. Unlike maltoside detergents in which alkyl chains were almost

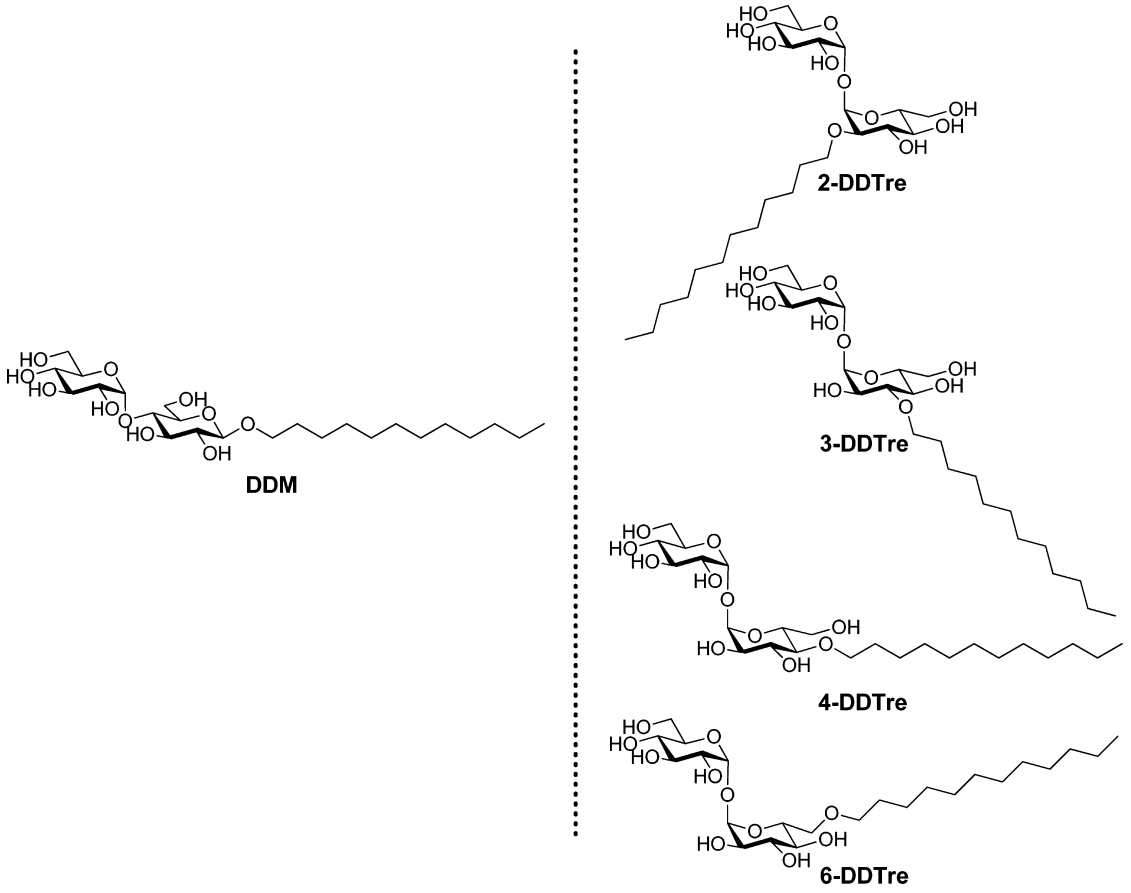
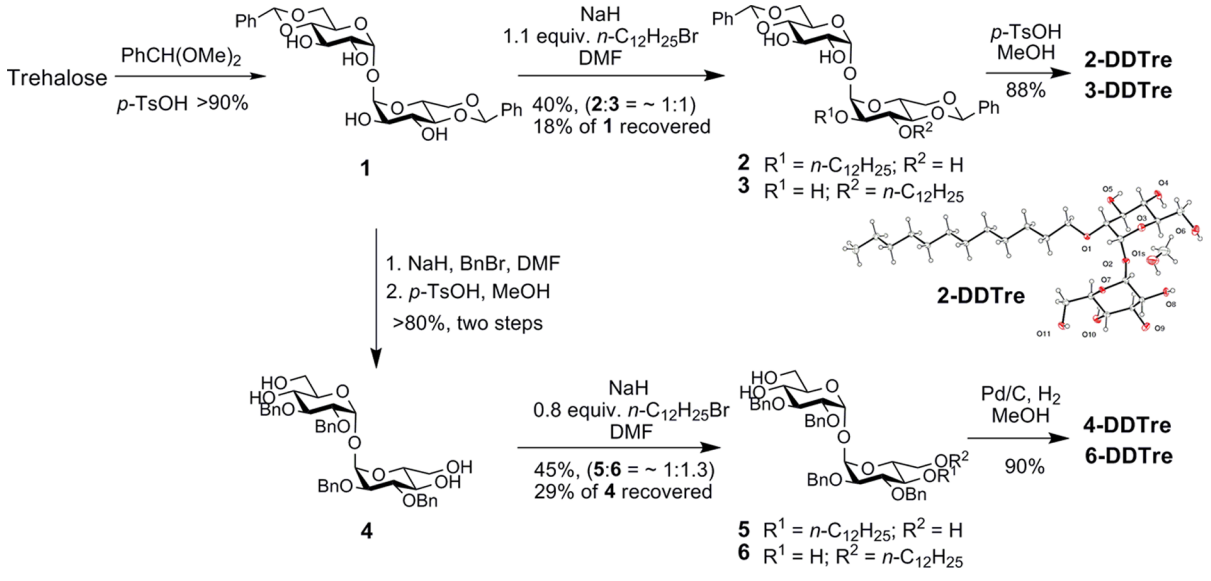


Figure 1. Chemical structures of DDM and trehalose-derived structural isomers: 2-, 3-, 4-, and 6-DDTre.

Scheme 1. Divergent Synthesis of Four *n*-Dodecyl Trehaloside Detergents, Each with the Alkyl Chain Attached to Different Positions of Trehalose[a]



[a]Embedded is the ORTEP drawing of the crystal structure of 2-DDTre in complex with one methanol molecule.

exclusively extended from the anomeric carbon of maltose through a convenient glycosidic bond formation,[28,29] trehaloside detergents need to be constructed by selectively attaching the alkyl chain through one of its four hydroxyl groups in the two-fold symmetric glucose unit using different chemistry. We set out to make *n*-dodecyl 2-, 3-, 4-, and 6-trehalosides as the analogue of DDM (Figure 1), one of the most popular detergents being used in membrane protein structural biology. The four dodecyl trehaloside (DDTre) molecules have the chemical composition and molecular weight identical to DDM yet with apparent distinction in molecular shape from each other, hence presenting structural diversity. Obviously, different protection/deprotection

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operations are necessary to construct 2-, 3-, 4-, and 6-DDTre separately, and achieving high regioselectivity of certain steps for the concise synthesis (e.g., for 2- and 3-DDTre) could be challenging. In this report, we devised a divergent strategy to reduce synthetic steps, which allowed us to quickly synthesize all four DDTre detergents in sufficient amounts for subsequent property evaluations.

Starting from 4,6:4′,6′-di-*O*-benzylidene trehalose **1**, we carried out a nonselective alkylation of the free 2- and 3-OH groups by reacting with 1.1 equiv of *n*-$C_{12}H_{25}Br$ in the presence of NaH in DMF at room temperature (Scheme 1). Precursors of 2-DDTre and 3-DDTre (**2** and **3**) were thus obtained as a ∼1:1 mixture in 40% yield, which ran as a single spot on either silica plates or column, but were well separated from the less polar multialkylated byproducts. Meanwhile, 18% of starting material (**1**) was recovered. Using more equivalents of *n*-$C_{12}H_{25}Br$ resulted in more byproducts rather than higher yield of the desired products. We also found that running the alkylation reaction at lower temperature increased the selectivity for **3** (e.g., **2**:**3** = 1:4 at 0 °C). For our purpose of accessing both 2- and 3-DDTre, the nonselective alkylation is ideal. Finally, benzylidene acetals of **2** and **3** were removed by acidic methanolysis to give 2- and 3-DDTre in 42% and 46% isolated yield, respectively, separated by silica chromatography. The close proximity of the dodecyl chain to the annomeric carbon (C1) in 2-DDTre, relative to other DDTre analogues, results in obvious separation of both ^{1}H and ^{13}C NMR signals for the two annomeric carbons and attached protons, as followed the structure assignment for 2-DDTre and 3-DDTre. The identity of 2-DDTre was also unambiguously confirmed by its crystal structure. Overall, 2- and 3-DDTre were each prepared in only three steps with an 18% total yield based on recovered starting material, and the reactions can be carried out in a multigram scale in a laboratory setting.

The strategic synthesis of 4- and 6-DDTre in a pair was similar to the above synthesis of 2- and 3-DDTre (Scheme 1). From the common intermediate **1**, tetrabenzylation of the remaining 2,2′,3,3′−OH groups and subsequent removal of 4,6- and 4′,6′-benzylidene acetals afforded the compound **4** in 80% yield. **4** was then reacted with 0.8 equivalent of *n*-$C_{12}H_{25}Br$ in the presence of NaH at room temperature to yield the monoalkylated products **5** and **6**, which were separated at this stage by silica chromatography (**5**:**6** = 1:1.3, 20% and 25% isolated yield, respectively) together with the recovery of 29% unreacted **4**. Subsequent removal of benzyl groups in both **5** and **6** by Pd/C-catalyzed hydrogenation gave 4-DDTre and 6-DDTre in 90% yield. Later, we also explored the synthesis of 4-DDTre alone because of its better performance for the stabilization of ORL1 (vide infra). Six steps, of which five steps for protection and deprotection operations, were performed to achieve a concise synthesis of 4-DDTre starting from trehalose (Scheme S1, SI). This synthesis route is one step longer than the concurrent synthesis of 4- and 6-DDTre outlined in Scheme 1 and may be further optimized for higher yield.

3.2. Micellar Properties of Trehaloside Detergents. The four structural isomers of DDTre display major differences in molecular geometry, which should have theoretical implication on the respective self-assembled micelle structures. We compared the following physical properties: solubility, hydrophobicity, CMC, and micelle size.

All four DDTre molecules are highly soluble in water (>10 wt %), except that the complete dissolution of 4-DDTre is a little slower at high concentrations. Interestingly, their hydrophobicity, evaluated by k', the normalized reversed phase (RP)-HPLC retention factor,[19] differs substantially from each other. All DDTre molecules are less hydrophobic than DDM, ranking in the order of 2-DDTre <3-DDTre <4-DDTre <6-DDTre < DDM (Figure S1, SI, k' = 1.92, 2.48, 2.99, 3.67, and 4.07 in the same order). The ranking of hydrophobicity, however, is unpredicted given their identical chemical compositions (identical polar and nonpolar segments for DDTre detergents). Of note, the evaluation is based on the assumption that each detergent runs as amonomer in the mixture of organic solvent and water (eluent: 50% acetonitrile in water) on a hydrophobic stationary phase. Indeed, we detected neither additional peaks on HPLC for each detergent being tested nor micelle structures (in the same solvent) by DLS measurements. It is possible that the decreased hydrophobicities of 2- and 3-DDTre relative to those of 4- and 6-DDTre and DDM is due to more effective hydration of the hydrophobic carbons proximal to the sugar moiety. In 2-DDTre (most hydrophilic), the alkyl chain is positioned close to the middle of two sugar units, whereas the alkyl chains are extended further away from the sugar hydroxyl groups in both 6-DDTre and DDM (most hydrophobic). In both 3- and 4-DDTre, the alkyl chains are instead proximal to two glucoside hydroxyl groups.

The CMC values of 2-DDTre, 3-DDTre, 4-DDTre, and 6-DDTre, measured in water using a fluorescence dye (ANS) incorporation assay, are 0.018% (0.35 mM), 0.024% (0.47 mM), 0.009% (0.18 mM), and 0.007% (0.14 mM), respectively (Figure 2). Since the formation of detergent micelles is driven mainly by hydrophobic association, the hydrophobicity is usually inversely correlated with the CMC value.[30] However, this is not exactly the case for DDTre molecules and DDM. For example, the CMCs for 4-DDTre, 6-DDTre, and DDM (0.008%, 0.16 mM) are about the same, but the hydrophobicity measurements differ largely as shown above. We assume that the interaction between polar groups also plays a significant role in the micelle formation of these sugar detergents.[31] We also noted that the ANS fluorescence curves in Figure 2 differ in the slope above the CMC of each detergent, which might indicate changes in total concentration and/or microenvironment of ANS molecules that partition into the different micelle solutions.

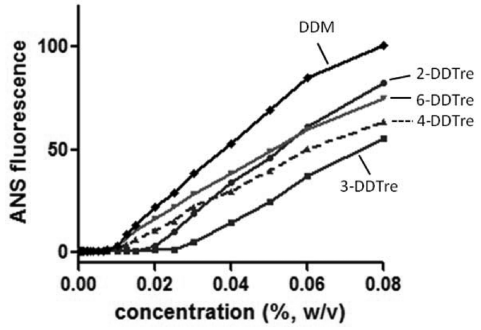

Figure 2. Changes of ANS fluorescence (λ_{ex} = 388 nm, λ_{em} = 477 nm) intensities with detergent concentrations. The CMC values are defined as the inflection point of fluorescence change.

The hydrodynamic radius (R_h) of micelles formed by each detergent was measured using dynamic light scattering (Figure 3). 3-DDTre, 6-DDTre, and DDM micelles are similar in size with mean radii of 3.3, 3.4, and 3.4 nm, respectively, and their size distributions are similar as well, which suggest a similar packing of monomers in the micelles for the three detergents. However, 2-DDTre forms much smaller micelles (R_h = 2.9 nm) indicative of a

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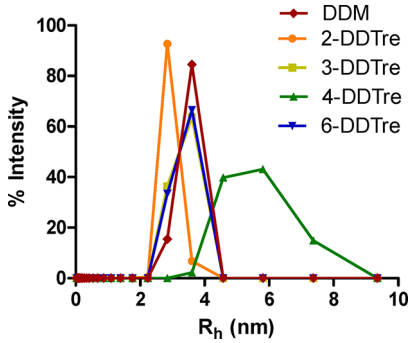

Figure 3. Hydrodynamic radii, R_h, of detergent micelles measured by dynamic light scattering. The data were an average of triplicate measurements for each detergent (0.2 wt % in water, 25 °C).

larger surface curvature. Interestingly, 4-DDTre forms the largest and most polydisperse micelles. The different micellar properties are a reflection of the distinct molecular geometry of the four DDTre and DDM molecules, and are potentially affected by hydrophilic interactions between the sugar units.

3.3. 2-DDTre Crystal Structure and Molecular Interaction in Crystalline States. Optical rotation and computational analysis indicate that the conformation of trehalose is relatively inflexible compared to most other disaccharides.[32,33] When comparing the multiple crystal structures of anhydrous trehalose and the trehalose dihydrate form, only small deviations were observed.[34−38] In this work, we have successfully grown 2-DDTre crystals in methanol at room temperature. Of note, no crystal structures of long alkyl maltosides and other disaccharide detergents have been found by searching the Cambridge Structural Database, which may be related to the flexible nature of these long-chain detergent structures. Our result is very intriguing also because methanol, a protic polar solvent like water, is often a superior solvent for the solubilization of long-chain hydrophobic detergents based on our experience. Alignment of the 2-DDTre structure with known crystal structures of anhydrous trehalose and trehalose dihydrate revealed relatively small deviations (Figure S2, SI), with calculated rmsd (root-mean-square deviation) values of 0.76 Å and 1.03 Å, respectively. The calculation is based on the distances of all C and O atoms of trehalose after overlay of the above structures. This analysis further supports the relatively inflexible conformation of trehalose, even after the modification of 2-OH group with a long alky chain that is proximal to the rotatable glycosidic linkage.

In the crystal structure, methanol was found in complex with 2-DDTre in 1:1 ratio, being packed in a polar channel formed by adjacent trehalosides in the symmetry-generated crystal packing (Figure 4). Overall, the crystal lattice displayed a pattern of alternating polar and nonpolar layers. The molecules approach from adjacent layers in a head-to-head (trehaloside) fashion with the alkyl chains angled at ∼65° (Figure 4b). The alkyl chains of 2-

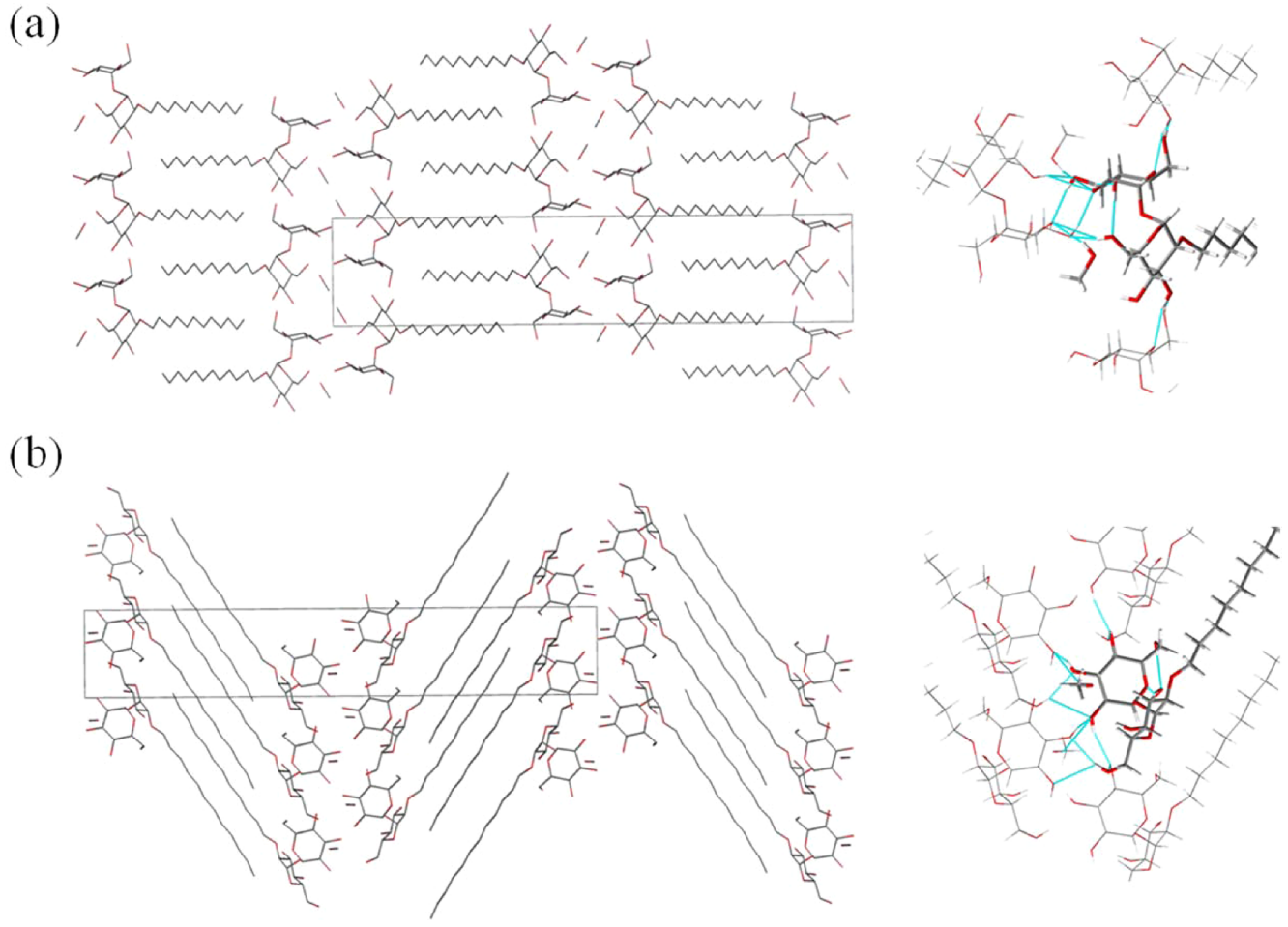

Figure 4. Crystal packing of 2-DDTre in complex with methanol, viewed along the (a) *a*-axis and the (b) *b*-axis of the unit cells (black box, left). On the right, zoomed-in structures, H-bond interactions among trehalosides are depicted by cyan lines.

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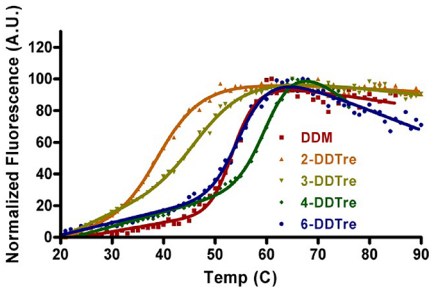

Detergent	T_m (°C)
DDM	53.9 ± 0.3
2-DDTre	41.4 ± 0.4
3-DDTre	50.1 ± 0.6
4-DDTre	61.4 ± 0.5
6-DDTre	55.7 ± 0.3

Figure 5. Thermal stability of ORL1 in the presence of various detergents (5 × CMC), probed by the CPM fluorescence thermal denaturation assay. Thermal transition temperatures (T_m) and standard errors were calculated by fitting the data to the equation as described previously.[21]

DDTre detergents adopt fully extended conformations and form interleaved, parallel sheets in each hydrophobic layer. In the polar layer, each trehaloside is connected to adjacent trehalosides with multiple H-bonds, as shown in the right, zoomed-in structures in Figure 4.

Although 2-DDTre was crystallized here in methanol, the crystal structure is useful for our understanding of the molecular interactions in an aqueous solution state.[39] We assume that the H-bonding among trehalosides plays a significant role in the formation of micelles. Accordingly, the H-bonding pattern for each DDTre detergent is likely different, thereby affecting the respective micelle properties as we have measured.

3.4. Solubilization and Stabilization of ORL1 and MsbA Using DDTre Detergents. DDM is the most popular commercial detergent for the solubilization of many human G-protein coupled receptors (GPCRs) and transporters. We evaluated the new DDTre detergents on the solubilization and stabilization of the nociceptin/orphanin FQ peptide receptor, ORL1, a member of opioid receptor family, and EcMsbA, a member of ATP-binding cassette (ABC) transporters. The structure of ORL1 containing the N-terminal fusion protein b_{562}RIL (BRIL-ORL) has most recently been determined using protein purified in DDM-cholesterol hemisuccinate (CHS) mixture and in meso phase crystallization technique.[40] Several MsbA homologue proteins including EcMsbA have been crystallized in maltoside detergents and structurally determined at moderate resolutions.[24]

In our test, all DDTre detergents behaved similarly as DDM for the extraction and solubilization of ORL1 from insect cell membranes (Figure S3, SI). The stability of ORL1 was subsequently assessed using a CPM fluorescence thermal denaturation assay that has been standardized in our laboratory for GPCR quality control measurement.[23] With this assay, cysteine residues buried inside proteins will become accessible to the CPM dye for covalent labeling upon thermal unfolding, and the thermal transition temperatures (T_m) are generally used as a measure of relative protein thermostability. We found that 4-DDTre was superior to other trehaloside isomers and DDM in its ability to thermally stabilize the receptor, giving the highest T_m at 61.4 °C (Figure 5). 6-DDTre (T_m = 55.7 °C) was comparable to DDM (T_m = 53.9 °C), whereas 2-DDTre and 3-DDTre were inferior (T_m = 41.4 and 50.1 °C, respectively). Similar stabilizing rank orders of the DDTre detergents were also observed on β2 adrenergic receptor (data not shown). It is interesting to note here that 4-DDTre, performing the best among the assessed detergents for stabilizing ORL1, forms the largest micelles with low CMC value, whereas 2-DDTre that destabilizes ORL1 forms the smallest micelles with high CMC (Figures 2 and 3). In addition, 6-DDTre and DDM have similar CMCs and micelle sizes, which also gave comparable ORL1 stability.

For MsbA purified in DDTre and maltoside detergents, we have measured the protein's enzymatic ATPase activity. The CPM thermal stability assay was not utilized because both cysteine residues (C88 and C315) in the wild-type construct of EcMsbA are not buried based on the X-ray structural model. Of note, DDM and UDM (β-D-undecylmaltoside), generally used for the purification and crystallization of MsbA, confer higher ATPase activity than most commercial detergents.[24,41] Our measurements showed that all four DDTre detergents could effectively extract and purify MsbA from cell membranes (Figure S4), and the high ATPase catalytic activity remained in all preparations (Figure 6). Further, EcMsbA purified in 2-DDTre

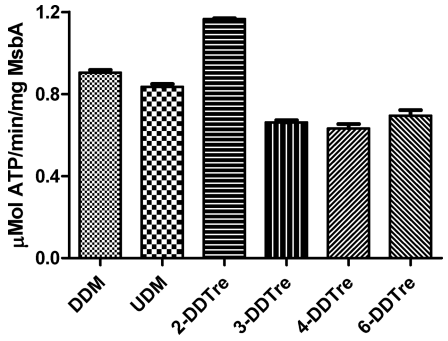

Figure 6. ATPase activity of EcMsbA in the presence of various detergents (3 × CMC), measured by a standard linked enzyme ATPase assay. The data are an average of three measurements with standard error bars shown.

gave relatively higher ATPase activity than DDM, UDM, and other DDTre analogues. No protein precipitation was observed in all the above detergent preparations after their incubation at 4 °C for more than two weeks.

That the detergent effect is protein dependent is a common observation. It is therefore not surprising that each DDTre detergent behaved differently in our tests on different (families of) proteins. Thus far, we still have very limited understanding of the detergent structure and function relationship with regards to their utility in membrane protein studies, and the detergent selection remains a more or less empirical process.[42] Nevertheless, our preliminary solubility and stability test of new trehaloside detergents on ORL1 and MsbA demonstrates that they are useful tools for membrane protein studies.

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4. CONCLUSION

The dodecyl trehalosides described in this report are structural isomers of DDM, which all contain identical alkyl chains at different positions on the sugar head but vary from each other in molecular shape and the respective micellar properties. Studies of these structural isomers and the various physicochemical properties underscore the importance of understanding the interactions between head groups/apolar tails for molecular assembly. Our results suggest that H-bond interactions among trehalosides and hydrophobic association in the apolar region, as revealed in the 2-DDTre crystal packing, may contribute together to DDTre micelle formation in aqueous solution. The forces mediating detergent self-association also likely affect how DDTre assembles onto membrane protein surfaces.

Our preliminary exploration of new trehaloside detergents demonstrates their utility as membrane protein solubilization reagents. The determination of their physicochemical properties is informational for future applications and possible structure and activity relationship analysis. In addition, trehalose detergents display several features that make their further development attractive. The exceptional chemical stability of trehalose compared to other disaccharides may be advantageous for the application of trehalose detergents in crystallization studies which often require weeks of incubation of proteins over a wide pH range. The stability and purity of chemical components is always a factor affecting the outcome of protein crystallization. In addition, the relative conformational inflexibility of trehalosides may also be advantageous for membrane protein crystallization. One of the major reasons that membrane proteins are difficult to crystallize is due to the high surface entropy of the flexible protein−detergent complexes. Therefore, limiting the flexibility of surface-associated detergents may benefit the crystal growth.[42] Moreover, given the well-known interactions between trehalose and phospolipids, investigation of trehalose detergents in lipid-based membrane protein platforms (e.g., bicelles, nanodiscs) is warranted.

■ ASSOCIATED CONTENT

Supporting Information

Supplemental figures, experimental procedure for the synthesis of 4-DDTre, NMR spectra for new compounds, and crystallographic information files (CIF) of 2-DDTre. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ REFERENCES

(1) Ohtake, S.; Wang, Y. J. Trehalose: Current use and future applications. *J. Pharm. Sci.* **2011**, *100*, 2020−2053.

(2) Sola-Penna, M.; Meyer-Fernandes, J. R. Stabilization against thermal inactivation promoted by sugars on enzyme structure and function: Why is trehalose more effective than other sugars? *Arch. Biochem. Biophys.* **1998**, *360*, 10−14.

(3) Kaushik, J. K.; Bhat, R. Why is trehalose an exceptional protein stabilizer? An analysis of the thermal stability of proteins in the presence of the compatible osmolyte trehalose. *J. Biol. Chem.* **2003**, *278*, 26458−26465.

(4) Diamant, S.; Eliahu, N.; Rosenthal, D.; Goloubinoff, P. Chemical chaperones regulate molecular chaperones in vitro and in cells under combined salt and heat stresses. *J. Biol. Chem.* **2001**, *276*, 39586−39591.

(5) Crowe, J. H. Trehalose as a "chemical chaperone": Fact and fantasy. *Adv. Exp. Med. Biol.* **2007**, *594*, 143−158.

(6) Crowe, J. H.; Crowe, L. M.; Jackson, S. A. Preservation of structural and functional activity in lyophilized sarcoplasmic reticulum. *Arch. Biochem. Biophys.* **1983**, *220*, 477−484.

(7) Crowe, L. M.; Mouradian, R.; Crowe, J. H.; Jackson, S. A.; Womersley, C. Effects of carbohydrates on membrane stability at low water activities. *Biochim. Biophys. Acta* **1984**, *769*, 141−150.

(8) Pereira, C. S.; Lins, R. D.; Chandrasekhar, I.; Freitas, L. C. G.; Huenenberger, P. H. Interaction of the disaccharide trehalose with a phospholipid bilayer: A molecular dynamics study. *Biophys. J.* **2004**, *86*, 2273−2285.

(9) Crowe, J. H.; Crowe, L. M.; Chapman, D. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. *Science* **1984**, *223*, 701−703.

(10) Goren, M. B. Sulfolipid I of Mycobacterium tuberculosis, strain H37Rv. II. Structural studies. *Biochim. Biophys. Acta* **1970**, *210*, 127−138.

(11) Brennan, P. J.; Nikaido, H. The envelope of mycobacteria. *Annu. Rev. Biochem.* **1995**, *64*, 29−63.

(12) Penkov, S.; Mende, F.; Zagoriy, V.; Erkut, C.; Martin, R.; Paessler, U.; Schuhmann, K.; Schwudke, D.; Gruner, M.; Maentler, J.; Reichert-Mueller, T.; Shevchenko, A.; Knoelker, H.-J.; Kurzchalia, T. V. Maradolipids: Diacyltrehalose glycolipids specific to dauer larva in Caenorhabditis elegans. *Angew. Chem., Int. Ed.* **2010**, *49* (9430−9435), S9430/1−S9430/10.

(13) Harland, C. W.; Rabuka, D.; Bertozzi, C. R.; Parthasarathy, R. The Mycobacterium tuberculosis virulence factor trehalose dimycolate imparts desiccation resistance to model mycobacterial membranes. *Biophys. J.* **2008**, *94*, 4718−4724.

(14) Harland, C. W.; Botyanszki, Z.; Rabuka, D.; Bertozzi, C. R.; Parthasarathy, R. Synthetic trehalose glycolipids confer desiccation resistance to supported lipid monolayers. *Langmuir* **2009**, *25*, 5193−5198.

(15) le Maire, M.; Champeil, P.; Moller, J. V. Interaction of membrane proteins and lipids with solubilizing detergents. *Biochim. Biophys. Acta* **2000**, *1508*, 86−111.

(16) Newstead, S.; Ferrandon, S.; Iwata, S. Rationalizing α-helical membrane protein crystallization. *Protein Sci.* **2008**, *17*, 466−472.

(17) Baddeley, T. C.; Wardell, J. L. Synthesis of per- and poly-substituted trehalose derivatives: studies of properties relevant to their use as excipients for controlled drug release. *J. Carbohydr. Chem.* **2009**, *28*, 198−221.

(18) Hough, L.; Palmer, A. K.; Richards, A. C. Chemical modification of trehalose. Part XI. 6,6′-dideoxy-6,6′-difluoro-α,α-trehalose and its galacto-analog. *J. Chem. Soc., Perkin Trans. 1* **1972**, 2513−2517.

(19) Armstrong, M. J.; Carey, M. C. The hydrophobic-hydrophilic balance of bile salts. Inverse correlation between reverse-phase high performance liquid chromatographic mobilities and micellar cholesterol-solubilizing capacities. *J. Lipid Res.* **1982**, *23*, 70−80.

(20) De Vendittis, E.; Palumbo, G.; Parlato, G.; Bocchini, V. A fluorimetric method for the estimation of the critical micelle concentration of surfactants. *Anal. Biochem.* **1981**, *115*, 278−286.

(21) Thompson, A. A.; Liu, J. J.; Chun, E.; Wacker, D.; Wu, H.; Cherezov, V.; Stevens, R. C. GPCR stabilization using the bicelle-like architecture of mixed sterol-detergent micelles. *Methods* **2011**, *55*, 310−307.

(22) Goto, Y.; Arai-Otsuki, S.; Tachibana, Y.; Ichikawa, D.; Ozaki, S.; Takahashi, H.; Iwasawa, Y.; Okamoto, O.; Okuda, S.; Ohta, H.; Sagara, T. Identification of a novel spiropiperidine opioid receptor-like 1 antagonist class by a focused library approach featuring 3D-pharmacophore similarity. *J. Med. Chem.* **2006**, *49*, 847−849.
(23) Alexandrov, A. I.; Mileni, M.; Chien, E. Y.; Hanson, M. A.; Stevens, R. C. Microscale fluorescent thermal stability assay for membrane proteins. *Structure* **2008**, *16*, 351−359.
(24) Ward, A.; Reyes, C. L.; Yu, J.; Roth, C. B.; Chang, G. Flexibility in the ABC transporter MsbA: Alternating access with a twist. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104*, 19005−19010.
(25) Vogel, G.; Steinhart, R. ATPase of Escherichia coli: purification, dissociation, and reconstitution of the active complex from the isolated subunits. *Biochemistry* **1976**, *15*, 208−216.
(26) Urbatsch, I. L.; Sankaran, B.; Weber, J.; Senior, A. E. P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site. *J. Biol. Chem.* **1995**, *270*, 19383−19390.
(27) Higashiyama, T. Novel functions and applications of trehalose. *Pure Appl. Chem.* **2002**, *74*, 1263−1269.
(28) Rosevear, P.; VanAken, T.; Baxter, J.; Ferguson-Miller, S. Alkyl glycoside detergents: a simpler synthesis and their effects on kinetic and physical properties of cytochrome c oxidase. *Biochemistry* **1980**, *19*, 4108−4115.
(29) Hong, W. X.; Baker, K. A.; Ma, X.; Stevens, R. C.; Yeager, M.; Zhang, Q. Design, synthesis, and properties of branch-chained maltoside detergents for stabilization and crystallization of integral membrane proteins: human connexin 26. *Langmuir* **2010**, *26*, 8690−8696.
(30) Tanford, C. *Hydrophobic Effect: Formation of Micelles and Biological Membranes*; John Wiley & Sons: New York, 1973.
(31) Venkatesan, P.; Cheng, Y.; Kahne, D. Hydrogen-bonding in micelle formation. *J. Am. Chem. Soc.* **1994**, *116*, 6955−6956.
(32) Duda, C. A.; Stevens, E. S. Trehalose conformation in aqueous-solution from optical-rotation. *J. Am. Chem. Soc.* **1990**, *112*, 7406−7407.
(33) Dowd, M. K.; Reilly, P. J.; French, A. D. Conformational-analysis of trehalose disaccharides and analogs using Mm3. *J. Comput. Chem.* **1992**, *13*, 102−114.
(34) Brown, G. M.; Rohrer, D. C.; Berking, B.; Beevers, C. A.; Gould, R. O.; Simpson, R. Crystal-Structure of α,α-trehalose dihydrate from 3 independent X-ray determinations. *Acta Crystallogr., Sect. B* **1972**, *28*, 3145−3158.
(35) Taga, T.; Senma, M.; Osaki, K. Crystal and molecular-structure of trehalose dihydrate. *Acta Crystallogr., Sect. B* **1972**, *28*, 3258−3263.
(36) Jeffrey, G. A.; Nanni, R. The crystal-structure of anhydrous α,α-trehalose at −150-degrees. *Carbohydr. Res.* **1985**, *137*, 21−30.
(37) Nagase, H.; Ogawa, N.; Endo, T.; Shiro, M.; Ueda, H.; Sakurai, M. Crystal structure of an anhydrous form of trehalose: Structure of water channels of trehalose polymorphism. *J. Phys. Chem. B* **2008**, *112*, 9105−9111.
(38) Stevens, E. D.; Dowd, M. K.; Johnson, G. P.; French, A. D. Experimental and theoretical electron density distribution of α,α-trehalose dihydrate. *Carbohydr. Res.* **2010**, *345*, 1469−1481.
(39) Chae, P. S.; Guzei, I. A.; Gellman, S. H. Crystallographic characterization of *N*-oxide tripod amphiphiles. *J. Am. Chem. Soc.* **2010**, *132*, 1953−1959.
(40) Thompson, A. A.; Liu, W.; Chun, E.; Katritch, V.; Wu, H.; Vardy, E.; Huang, X.-P.; Trapella, C.; Guerrini, R.; Calo, G.; Roth, B. L.; Cherezov, V.; Stevens, R. C. Structure of the nociceptin/orphanin FQ receptor in complex with a peptide mimetic. *Nature* **2012**, *485*, 395−399.
(41) Zhang, Q.; Ma, X.; Ward, A.; Hong, W. X.; Jaakola, V. P.; Stevens, R. C.; Finn, M. G.; Chang, G. Designing facial amphiphiles for the stabilization of integral membrane proteins. *Angew. Chem., Int. Ed.* **2007**, *46*, 7023−7025.
(42) Zhang, Q.; Tao, H.; Hong, W. X. New amphiphiles for membrane protein structural biology. *Methods* **2011**, *55*, 318−323.