Synthesis and Properties of Dodecyl Trehaloside Detergents for Membrane Protein Studies

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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-carboned 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 analog, β- D-dodecylmaltoside (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 a,a-trehalose, is an atypical, non-reducing disaccharide composed of two D-glucoses with an a,a-1,1′-glycosidic linkage. The non-reducing 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 additive reagents 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 non-covalent 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 dehydration and rehydration process, an effect unsurmounted by other sugars.[5–7] Related, 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 replacing 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]

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 maltose head group, prevail in the solubilization, purification, stabilization and structural studies of membrane proteins, in particular for challenging eukaryotic targets.[16] The prevalence of non-charged maltoside detergents can be attributed to their relatively mild, non-denaturing properties and their increasing success in highly demanding crystallization efforts. It is worthwhile to emphasize that the selection of a detergent molecule, 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, 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

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$_\alpha$ radiation (Bruker FR-591 Rotating Anode Generator/Montels Optics λ = 1.5478). *n*-Dodecyl *β*-D-maltoside (DDM) was purchased from Anatrace.

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$_2$SO$_4$, filtered, and concentrated in vacuo. The residue was purified by silica gel chromatography (eluent: hexanes/EtOAc = 3/1 and 100% acetone), which gave a non-separable 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 pre-absorbed 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$_3$OD) 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$_3$OD) 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$_{47}$O$_{11}$Na ([M + Na]$^+$) 533.3, found 533.3. 3-DDTre: ^{1}H NMR (500 MHz, CD$_3$OD) 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$_3$OD) 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.

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 (**2**) 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 with *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$_2$SO$_4$, 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** were reported in Supporting Information. 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$_3$OD) 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$_3$OD) 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$_{47}$O$_{11}$Na ([M + Na]$^+$) 533.3, found 533.3. 6-DDTre: ^{1}H NMR (500 MHz, CD$_3$OD) 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), 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$_3$OD) 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$_{47}$O$_{11}$Na ([M + Na]$^+$) 533.3, found 533.3.

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 5μ, 150 × 4.60 mm) and a mobile phase of 50% CH$_3$CN/50% H$_2$O at a flow rate of 1.0 mL/min. Assignment of each detergent peak was confirmed by multiple single and co-injections. 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]

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.

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.

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 haemagglutinin 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(3H),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 hour. Iodoacetamide (Sigma) was then added to the membranes at a final concentration of 1 mg/mL for another 15 minutes before solubilization with 0.5% (w/v) each detergent being tested for three hours at 4 °C. The supernatant was isolated by centrifugation at $160,000 \times g$ for 45 minutes, 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 $5 \times CMC$ 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 $5 \times CMC$ of each detergent being tested. The eluted protein in each detergent was assayed for purity by SDS gel electrophoresis.

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 $5 \times CMC$ of each detergent being tested. Fluorescence emission was measured on a Cary Eclipse Fluorescence Spectrophotometer ($\lambda_{ex} = 387$ nm; $\lambda_{em} = 463$ 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 non-linear regression analysis (GraphPad Prism) of fluorescence signal versus T plots according to equation described previously.[21]

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, 0.1mg/ml of DNase I, and proteinase inhibitor cocktail. The supernatant after centrifugation at $38,000g$ for 45 min was subject to Ni-affinity column for purification. $3 \times CMC$ of each detergent being tested was included in the wash and elution steps. MsbA was further purified by desalting column using buffer containing $3 \times CMC$ of each detergent, 20 mM Tris (pH 7.5) and 20 mM NaCl. The protein purity was assessed by SDS gel electrophoresis.

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 340nm 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

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 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 2-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 a structural diversity. Obviously, different protection/deprotection operations are necessary to construct 2-, 3-, 4- and 6-DDTre separately, and achieving high regioselectivity for 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 amount for subsequent property evaluations.

Starting from 4,6:4′,6′-di-*O*-benzylidene trehalose **1**, we carried out a non-selective alkylation of the free 2- and 3-OH groups by reacting with 1.1 equivalent of $n\text{-}C_{12}H_{25}Br$ 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 were run as a single spot on either silica plates or column but well separated from the less polar multi-alkylated byproducts. Meanwhile, 18% of starting material (**1**) was recovered. Using higher equivalents of $n\text{-}C_{12}H_{25}Br$ 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 to both 2- and 3-DDTre, the non-selective alkylation is ideal. Finally, benzylidene acetals of **2** and **3** were removed by acidic methanolysis to give 2- and 3-DDTre in 40% and 42% 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 analogs, 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 18% total yield based on the recovered starting material, starting from trehalose in only three steps, and the reactions can be carried out in multigram scales 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 $n\text{-}C_{12}H_{25}Br$ in the presence of NaH at room temperature to yield the mono-alkylated 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). 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.

Micellar Properties of Trehaloside Detergents

The four structural isomers of DDTre display major differences in molecular geometry, which is supposed to affect 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, 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 monomers in the mixture of organic solvent and water (eluent: 50% acetonitrile in water) on a hydrophobic stationary phase. Indeed, we detected neither additional peak on HPLC for every detergent being tested nor micelle structures (in the same solvent) by DLS measurements. Possibly, the decreased hydrophobicity of 2- and 3-DDTre relative to 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 proximal to two glucoside hydroxyl groups instead.

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.

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 distribution is 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 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.

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 a small deviation was found from each other.[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 showed relatively small deviation (Figure S2), 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-DDTre detergents adopt fully extended conformation 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.

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 the protein material purified in the DDM-cholesterol hemisuccinate (CHS) mixture and *in meso* phase crystallization technique.[40] Several MsbA homolog 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). 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 gave relatively higher ATPase activity than DDM, UDM, and other DDTre analogs. No protein precipitation was observed in all 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.

4. Conclusion

The dodecyl trehalosides described in this report are structural isomers of DDM, which all contain identical alkyl chains at different positions of a sugar head but vary from each other in molecular shape and the respective micellar properties. Studies of these structural isomers and the various physico-chemical 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 the DDTre micelle formation in aqueous solution. The forces mediating detergent self-association also likely affect how they assemble onto membrane protein surfaces.

Our preliminary test of new trehaloside detergents demonstrates their utility as membrane protein solubilization reagents. The determination of their physico-chemical 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.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

References

1. Ohtake S, Wang YJ. Trehalose: Current use and future applications. J. Pharm. Sci. 2011; 100:2020–2053. [PubMed: 21337544]

2. Sola-Penna M, Meyer-Fernandes JR. 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. [PubMed: 9826423]

3. Kaushik JK, 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. [PubMed: 12702728]

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. [PubMed: 11517217]

5. Crowe JH. Trehalose as a "chemical chaperone" fact and fantasy. Adv. Exp. Med. Biol. 2007; 594:143–158. [PubMed: 17205682]

6. Crowe JH, Crowe LM, Jackson SA. Preservation of structural and functional activity in lyophilized sarcoplasmic reticulum. Arch. Biochem. Biophys. 1983; 220:477–484. [PubMed: 6824335]

7. Crowe LM, Mouradian R, Crowe JH, Jackson SA, Womersley C. Effects of carbohydrates on membrane stability at low water activities. Biochim. Biophys. Acta. 1984; 769:141–150. [PubMed: 6691971]

8. Pereira CS, Lins RD, Chandrasekhar I, Freitas LCG, Huenenberger PH. Interaction of the disaccharide trehalose with a phospholipid bilayer: a molecular dynamics study. Biophys. J. 2004; 86:2273–2285. [PubMed: 15041666]

9. Crowe JH, Crowe LM, Chapman D. Preservation of membranes in anhydrobiotic organisms: the role of trehalose. Science. 1984; 223:701–703. [PubMed: 17841031]

10. Goren MB. Sulfolipid I of Mycobacterium tuberculosis, strain H37Rv. II. Structural studies. Biochim. Biophys. Acta. 1970; 210:127–138. [PubMed: 4989542]

11. Brennan PJ, Nikaido H. The envelope of mycobacteria. Annu. Rev. Biochem. 1995; 64:29–63. [PubMed: 7574484]

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 TV. Maradolipids: Diacyltrehalose Glycolipids Specific to Dauer Larva in Caenorhabditis elegans. Angew. Chem. Int. Ed. 2010; 49:9430–9435. S9430/1–S9430/10.

13. Harland CW, Rabuka D, Bertozzi CR, Parthasarathy R. The Mycobacterium tuberculosis virulence factor trehalose dimycolate imparts desiccation resistance to model mycobacterial membranes. Biophys. J. 2008; 94:4718–4724. [PubMed: 18326657]

14. Harland CW, Botyanszki Z, Rabuka D, Bertozzi CR, Parthasarathy R. Synthetic trehalose glycolipids confer desiccation resistance to supported lipid monolayers. Langmuir. 2009; 25:5193–5198. [PubMed: 19323499]

15. le Maire M, Champeil P, Moller JV. Interaction of membrane proteins and lipids with solubilizing detergents. Biochim. Biophys. Acta. 2000; 1508:86–111. [PubMed: 11090820]

16. Newstead S, Ferrandon S, Iwata S. Rationalizing alpha-helical membrane protein crystallization. Protein Sci. 2008; 17:466–472. [PubMed: 18218713]

17. Baddeley TC, Wardell JL. 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 AK, Richards AC. Chemical Modification of Trehalose .9. 6,6'- Dideoxy-6,6'- Difluoro-Alpha Alpha-Trehalose and Its Galacto-Analog. J. Chem. Soc. Perk. Trans. 1. 1972 2513-&.

19. Armstrong MJ, Carey MC. 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. [PubMed: 7057113]

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. [PubMed: 7304960]

21. Thompson AA, Liu JJ, Chun E, Wacker D, Wu H, Cherezov V, Stevens RC. GPCR stabilization using the bicelle-like architecture of mixed sterol-detergent micelles. Methods. 2011; 55:310–317. [PubMed: 22041719]

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. [PubMed: 16451050]

23. Alexandrov AI, Mileni M, Chien EY, Hanson MA, Stevens RC. Microscale fluorescent thermal stability assay for membrane proteins. Structure. 2008; 16:351–359. [PubMed: 18334210]

24. Ward A, Reyes CL, Yu J, Roth CB, Chang G. Flexibility in the ABC transporter MsbA: Alternating access with a twist. Proc. Natl. Acad. Sci. U.S. A. 2007; 104:19005–19010. [PubMed: 18024585]

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. [PubMed: 2281]

26. Urbatsch IL, Sankaran B, Weber J, Senior AE. P-glycoprotein is stably inhibited by vanadate-induced trapping of nucleotide at a single catalytic site. J. Biol. Chem. 1995; 270:19383–19390. [PubMed: 7642618]

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. [PubMed: 6250583]

29. Hong WX, Baker KA, Ma X, Stevens RC, 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. [PubMed: 20232919]

30. Tanford, C. Hydrophobic Effect: Formation of Micelles and Biological Membranes. New York: John Wiley & Sons; 1973.

31. Venkatesan P, Cheng Y, Kahne D. Hydrogen-Bonding in Micelle Formation. J. Am. Chem. Soc. 1994; 116:6955–6956.

32. Duda CA, Stevens ES. Trehalose Conformation in Aqueous-Solution from Optical-Rotation. J. Am. Chem. Soc. 1990; 112:7406–7407.

33. Dowd MK, Reilly PJ, French AD. Conformational-Analysis of Trehalose Disaccharides and Analogs Using Mm3. J. Comput. Chem. 1992; 13:102–114.

34. Brown GM, Rohrer DC, Berking B, Beevers CA, Gould RO, Simpson R. Crystal-Structure of Alpha,Alpha-Trehalose Dihydrate from 3 Independent X-Ray Determinations. Acta Crystallogr. B. 1972; B 28:3145–3158.

35. Taga T, Senma M, Osaki K. Crystal and Molecular-Structure of Trehalose Dihydrate. Acta Crystallogr. B. 1972; B 28:3258–3263.

36. Jeffrey GA, Nanni R. The Crystal-Structure of Anhydrous Alpha,Alpha-Trehalose at - 150-Degrees. Carbohydr. Res. 1985; 137:21–30. [PubMed: 3986848]

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. [PubMed: 18605683]

38. Stevens ED, Dowd MK, Johnson GP, French AD. Experimental and theoretical electron density distribution of alpha,alpha-trehalose dihydrate. Carbohydr. Res. 2010; 345:1469–1481. [PubMed: 20381017]

39. Chae PS, Guzei IA, Gellman SH. Crystallographic characterization of N-oxide tripod amphiphiles. J. Am. Chem. Soc. 2010; 132:1953–1959. [PubMed: 20095541]

40. Thompson AA, Liu W, Chun E, Katritch V, Wu H, Vardy E, Huang X-P, Trapella C, Guerrini R, Calo G, Roth BL, Cherezov V, Stevens RC. Structure of the Nociceptin/Orphanin FQ Receptor in Complex with a Peptide Mimetic. Nature. 2012; 485:395–399. [PubMed: 22596163]

41. Zhang Q, Ma X, Ward A, Hong WX, Jaakola VP, Stevens RC, Finn MG, Chang G. Designing facial amphiphiles for the stabilization of integral membrane proteins. Angew. Chem. Int. Ed. Engl. 2007; 46:7023–7025. [PubMed: 17691085]

42. Zhang Q, Tao H, Hong WX. New amphiphiles for membrane protein structural biology. Methods. 2011; 55:318–323. [PubMed: 21958988]
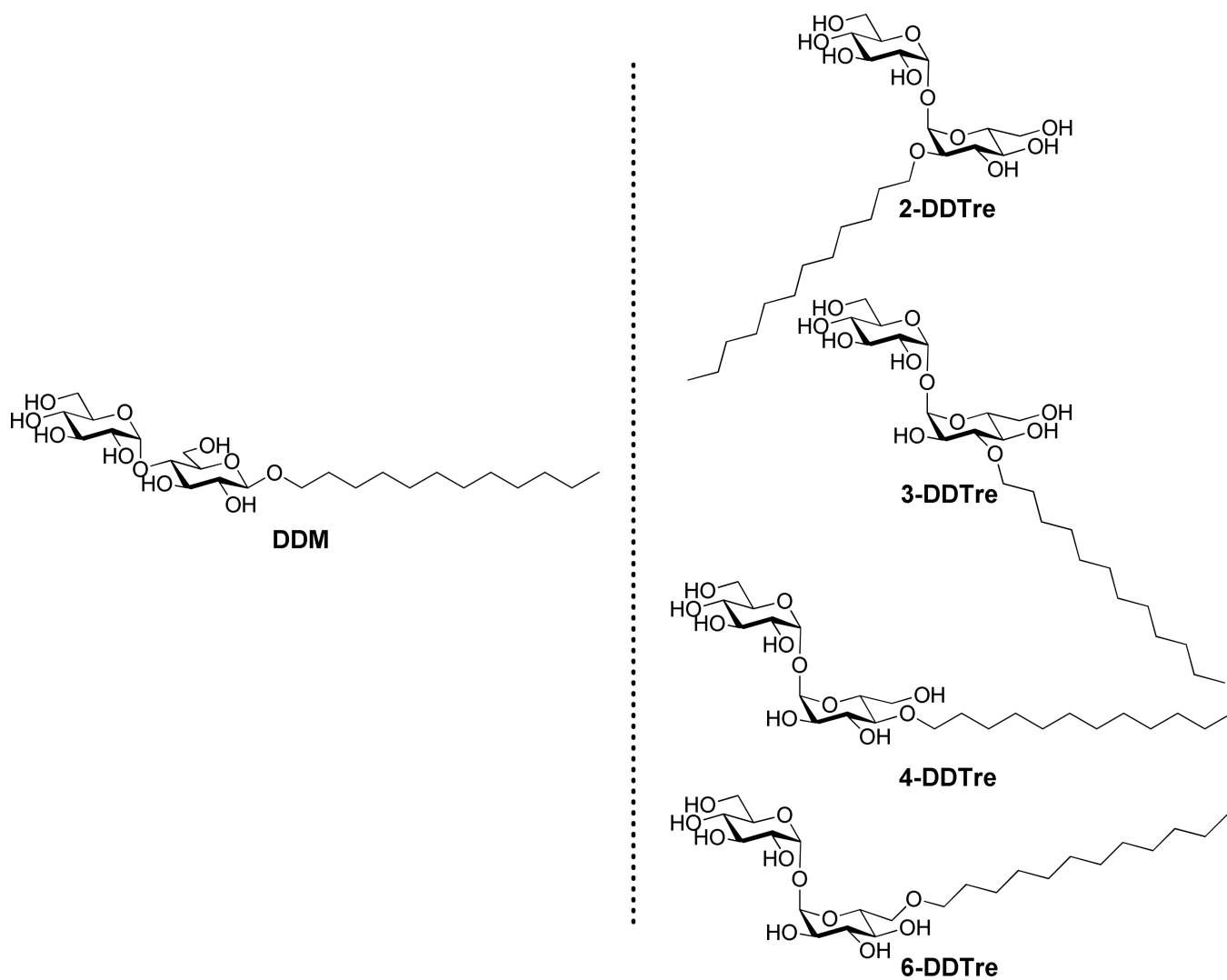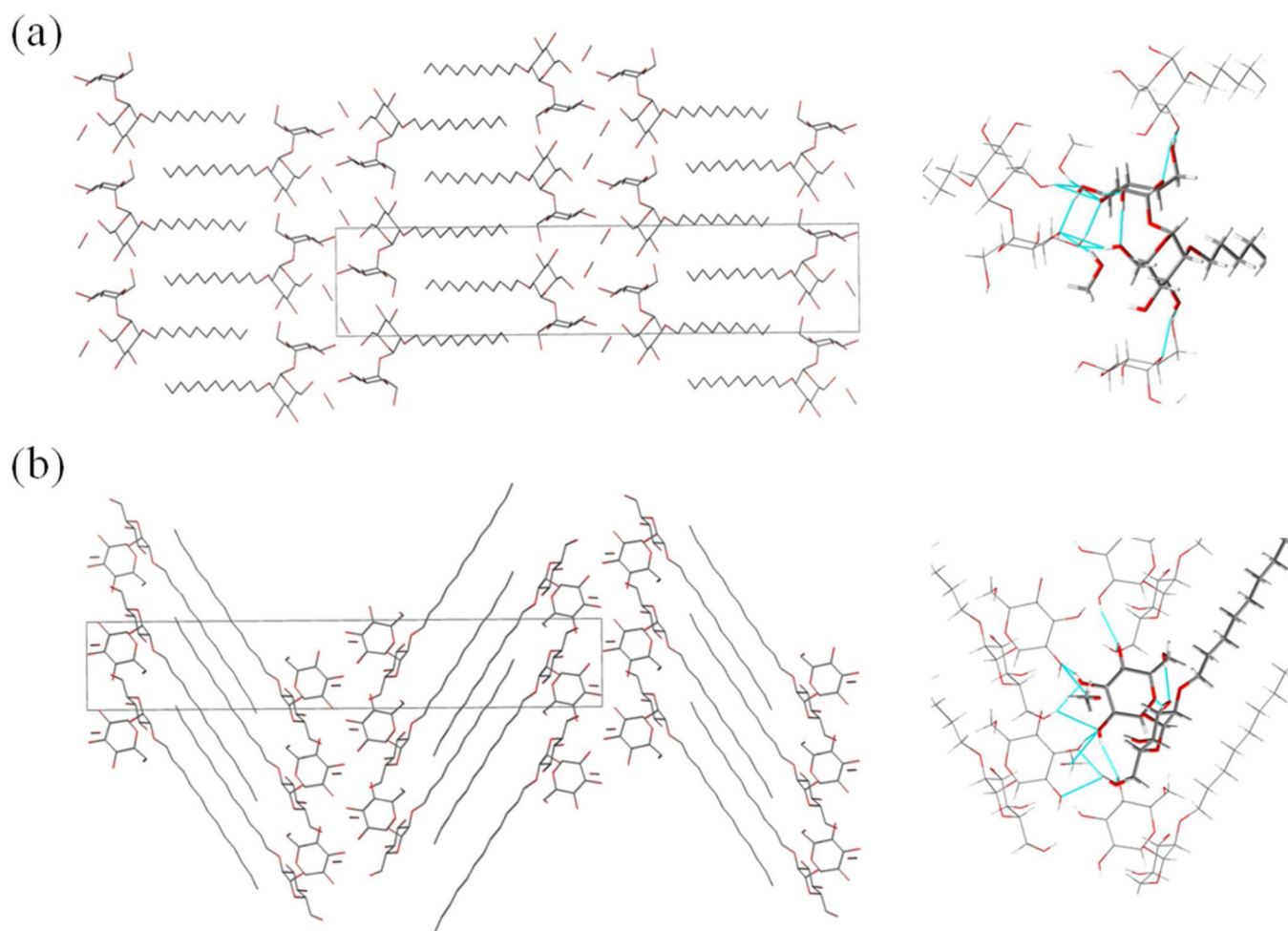
Figure 1.
Chemical structures of DDM and trehalose-derived structural isomers: 2-, 3-, 4-, and 6-DDTre.
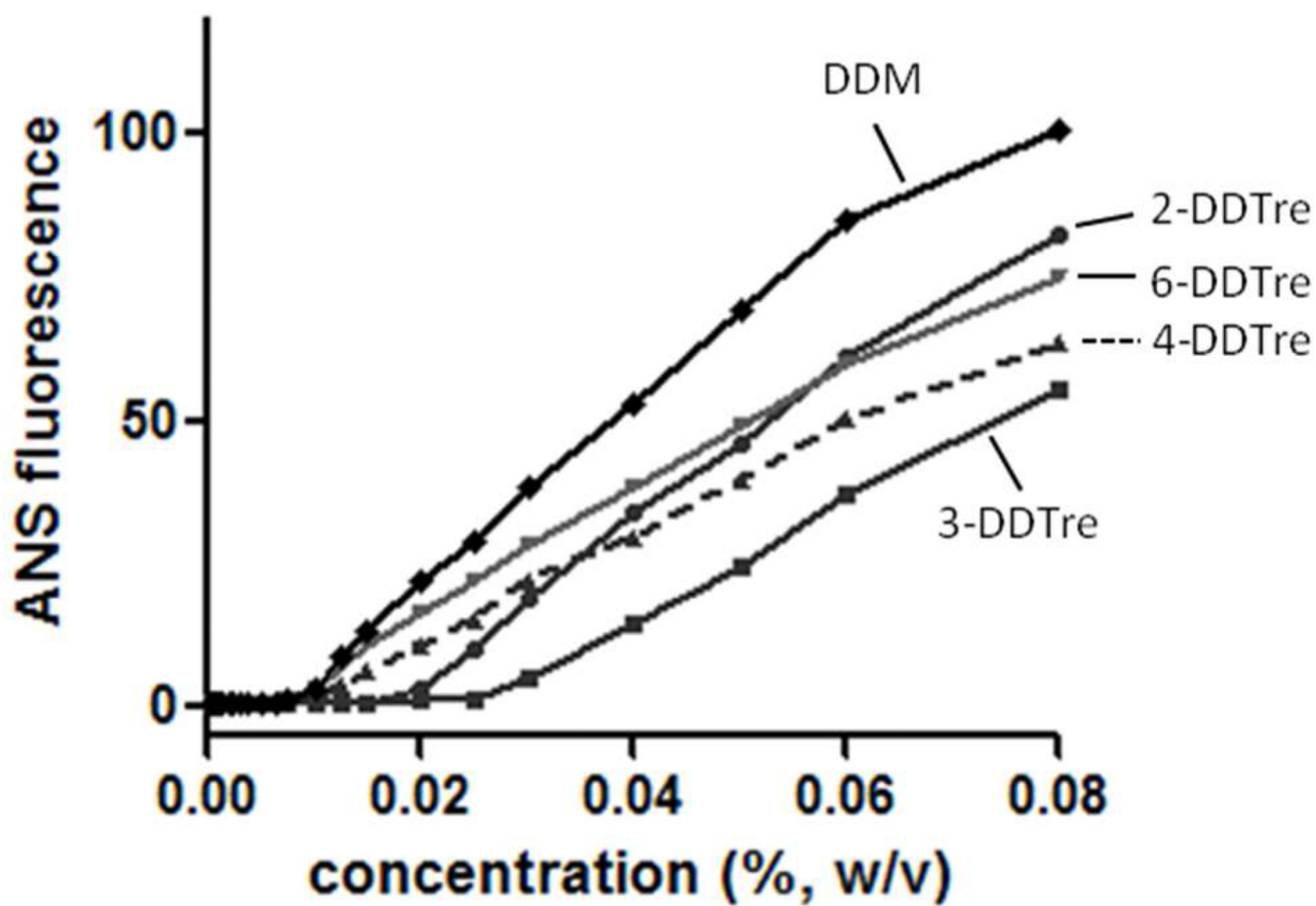
Figure 2.
Changes of ANS fluorescence (λ_{ex}= 388 nm, λ_{em} = 477 nm) intensities with detergent concentrations. The CMC values are defined as the inflection point of fluorescence change.
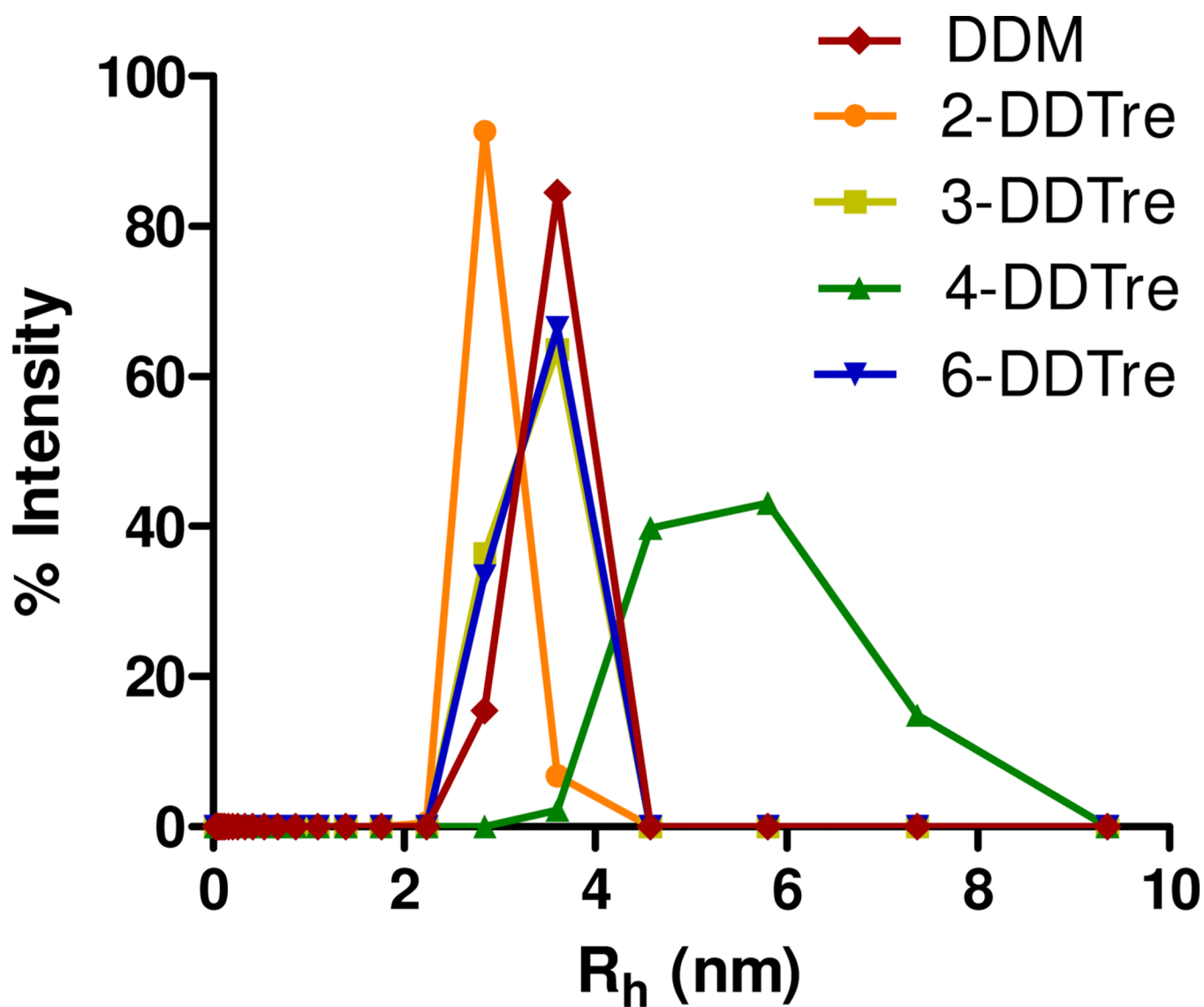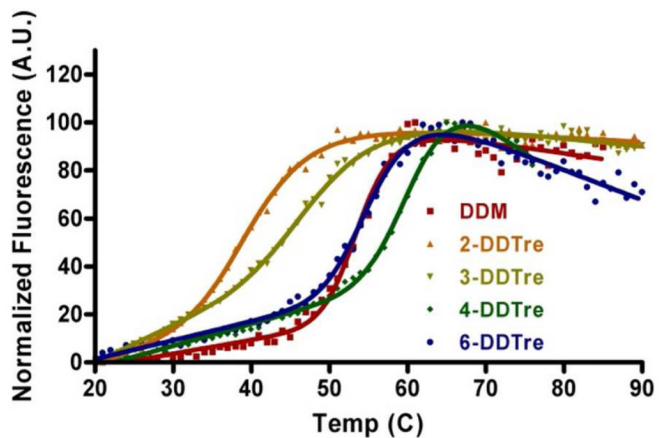
Figure 3.
Hydrodynamic radii, R_h, of detergent micelles measured by dynamic light scattering. The data were an average of triplicate measurements for each detergent (0.2 wt % in water, 25 °C).
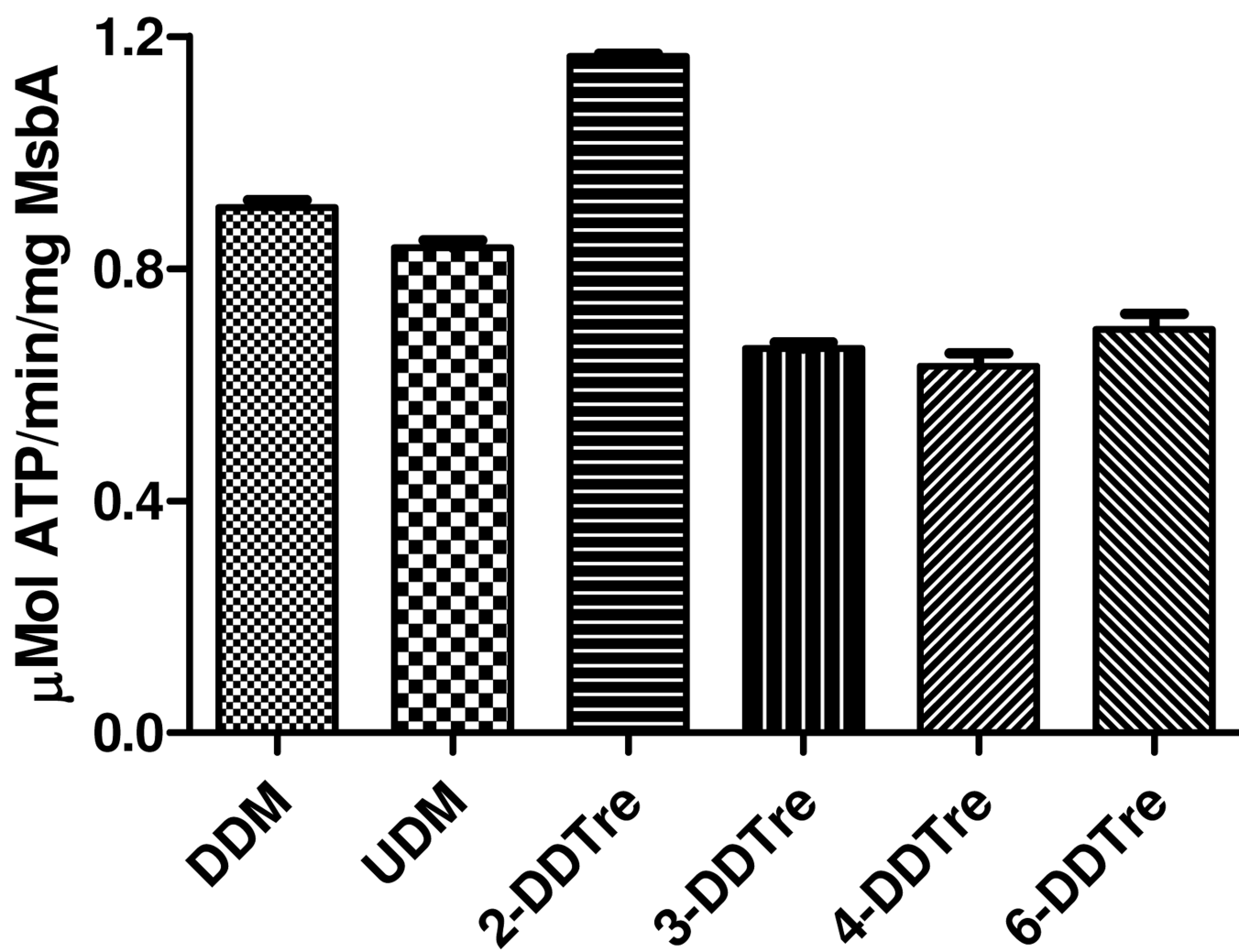
Figure 4.
Crystal packing of 2-DDTre in complex with methanol, viewed along (a) a-axis and (b) b-axis of the unit cells (black box, left). On the right, zoomed-in structures, H-bond interactions among trehalosides are depicted by cyan lines.
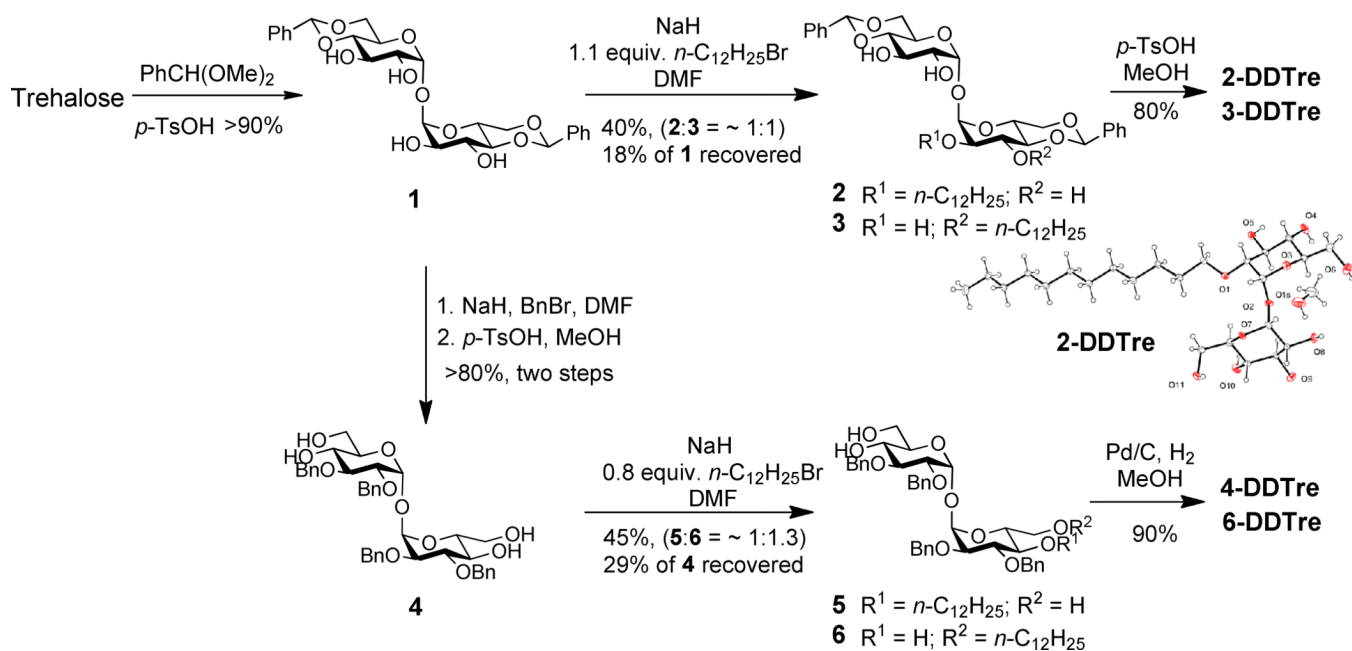
Detergent	T_m (°C)
DDM	53.9 ± 0.3
2-DDTre	41.4 ± 0.4
3-DDTre	50.1 ± 0.6
4-DDTre	61.4 ± 0.5
6-DDTre	55.7 ± 0.3

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

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

Scheme 1.
Divergent synthesis of four *n*-dodecyl trehaloside detergents, each with the alkyl chain attached to different positions of trehalose. Embedded is the ORTEP drawing of the crystal structure of 2-DDTre in complex with one methanol molecule.