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Steroid-based amphiphiles for membrane protein study: Importance of alkyl spacer for protein stability

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Abstract

Membrane proteins allow effective communication between cells and organelles and their external environments. Maintaining membrane protein stability in a non-native environment is the major bottleneck to their structural study. Detergents are widely used to extract membrane proteins from the membrane and keep the extracted protein in a stable state for downstream characterization. In the current study, three sets of steroid-based amphiphiles, glyco-diosgenin analogs (GDNs), steroid-based penta-saccharides either lacking a linker (SPSs) or with a linker (SPS-Ls), were developed as novel chemical tools for membrane protein research. These detergents were tested with three membrane proteins in order to characterize their ability to extract membrane proteins from the membrane and to stabilize membrane proteins long term. Some of the novel detergents, particularly the SPS-Ls, displayed favorable behaviors with the tested membrane proteins. This result indicates the potential utility of these detergents as chemical tools for membrane protein structural study and a critical role of the simple alkyl spacer in determining detergent efficacy.

Graphical Abstract

New steroid amphiphiles with a penta-saccharide head group displayed favourable behaviour for membrane protein stability. This study indicates an important role of a flexible alkyl spacer in determining detergent efficacy for protein stabilization.

Keywords

novel amphiphiles; membrane proteins; molecular design; protein stability; steroid

Introduction

Membrane proteins are encoded by approximately 30% of the human genome and are vitally important for a number of cellular functions.¹ Membrane transporters and channels regulate material transfer while membrane receptors are responsible for signal transduction and cell-cell communication. Due to these crucial roles in cell physiology, dysfunctions of membrane proteins are directly implicated in a wide range of human diseases, and they are resultantly potential therapeutic targets; more than half of pharmaceuticals target membrane proteins.² The structural and functional studies of these proteins are far more difficult than those of soluble proteins as they tend to aggregate or denature in a non-lipidic environment. The planar architecture of lipid bilayers is most suitable for protein stability as this arrangement exerts a stronger lateral pressure on the membrane proteins compared to detergent micelles. In addition, some membrane lipids (*e.g.*, cholesterol) are specifically associated with membrane protein surfaces and have roles in protein function.^{3–5} However, for downstream characterization, these bio-macromolecules have to be extracted from the membranes. Thus, we need a membrane mimetic system that preserves the native structures of membrane proteins in non-native environments.

Detergent micelles with globular or elliptical architecture are popularly used as a membrane mimetic system. These self-assemblies formed by amphipathic molecules with conical geometry possess the ability to extract/solubilize membrane proteins from the membranes.⁶ These amphipathic molecules are also used to maintain the structures and functions of target proteins over the course of protein solubilization, purification and crystallization. Of the more than 120 conventional detergents, only a handful of detergents, as exemplified by *n*-octyl- β -D-glucoside (OG), *n*-decyl- β -D-maltoside (DM) and *n*-dodecyl- β -D-maltoside (DDM), are widely used for membrane protein manipulation.⁶ These conventional detergents typically comprise a single flexible alkyl chain and a relatively large head group.⁸ Some detergents such as those in the 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS) and Tween series, substantially deviate from this classical architecture, and are known to be suitable for only a small number of membrane proteins.⁹ Membrane proteins solubilized even in the popular detergents (*e.g.*, DDM) can lose

structural integrity over the course of protein extraction and purification, hampering advances in membrane protein research.¹⁰ Because of their diverse roles in cellular function and highly variable 3D structures, membrane proteins differ greatly in architecture and properties. Such variability is not reflected in conventional detergents. The large gap in diversity of membrane proteins and the chemical tools available to study them, limits membrane protein structural study. Therefore, it is necessary to develop distinctive new amphiphiles with enhanced efficacy for membrane protein stabilization.

Over the past two decades, dozens of novel amphiphilic agents have been developed to solve the issues associated with conventional detergents. Polymeric amphiphiles such as amphipols (Apols)¹¹ and styrene maleic acid (SMA) copolymers, and nanodiscs (NDs) proved to be effective for protein structural studies *via* nuclear magnetic resonance (NMR) spectroscopy and electron microscopy (EM).¹² However, to date, these agents have only had limited success in terms of high resolution X-ray crystallographic membrane protein structures and are often suboptimal for membrane protein extraction. Small amphipathic agents, in contrast, are widely used because they are not only efficient at extracting membrane proteins, but are also effective at preserving structural integrity during membrane protein purification and crystallization. Representatives include hemifluorinated surfactants (HFSs),¹³ cholate- or resorcinarene-based facial amphiphiles (FAs and RGAs),^{14,15} steroid-based amphiphiles (*e.g.*, chobimalt¹⁶ and GDN¹⁷) and neopentyl glycol (NG) amphiphiles (GNGs and MNGs),^{18–20} penta-saccharide-based amphiphiles (PSEs),²¹ mannitol-based amphiphiles (MNAs),²² neopentyl glycol-derived triglucosides (NDTs),²³ dendronic trimaltosides (DTMs),²⁴ and glycosyl-substituted dicarboxylate detergents (DCODs).²⁵ Among these small amphipathic agents, steroid-based amphiphiles such as chobimalt and GDN have received attention because cholesterol is an important component of eukaryotic membranes (Figure S1), and use of its derivatives (*e.g.*, cholesteryl hemisuccinate (CHS)) as additives can improve protein stability through protein surface binding.²⁶ In this context, it is encouraging that GDN has very recently facilitated high resolution structural determination of two challenging membrane proteins *via* cryo-EM,²⁷ indicating that multi-fused ring-bearing steroids have use as detergent hydrophobic groups. Herein, we prepared three sets of steroid-based amphiphiles and tested with a few membrane proteins to evaluate their efficacy for protein stabilization. One set contains two maltoside head groups directly connected onto diosgenin hydrophobic group, designated GDN analogs, while the other two sets contain a penta-saccharide group connected with four different steroidal groups either directly (steroid-based penta-saccharides (SPSs)) or *via* a linker (SPS-Ls). Some of these agents, particularly the SPS-Ls, conferred enhanced stability to a couple of membrane proteins including a G-protein coupled receptor (GPCR), compared to a gold standard conventional detergent (DDM).

Results and Discussion

The new agents feature a rigid steroid-based lipophilic group and a carbohydrate-based hydrophilic group. The first novel detergents are GDN variants containing diosgenin as a lipophilic group and two maltoside head groups. The maltose head groups were directly attached to the lipophilic group *via* two hydroxyl groups introduced to the first/second ring (A/B ring).¹⁷ GDN-1 and GDN-2 have two maltoside groups attached to two neighboring

carbons (C2 and C3) of the lipophilic group (*i.e.*, diosgenin) (Scheme 1). In the case of GDN-3, these head groups were introduced into two non-neighboring carbons (C3 and C6) of the lipophilic group. Thus, GDN-1/2 has the head groups well segregated from the diosgenin lipophilic group, and are thus highly amphiphilic, while GDN-3 has a relatively small degree of amphiphilicity due to the reduced segregation between the hydrophilic and lipophilic groups (see the Newman projections in Scheme 1). GDN-1 and GDN-2 differ from each other in relative orientation of the two maltoside groups; the maltoside groups of GDN-1 are directed to an opposite side of the ring (*axial-axial*) whereas those of GDN-2 are in the same side of the ring (*axial-equatorial*). The relative locations and orientations of two maltoside groups of the GDNs were schematically represented in the individual Newman projections in Scheme 1. These GDNs share the diosgenin lipophilic unit and two maltoside groups with the previously developed GDN, but differ from the previous agent in that they contain a direct head-to-tail connection without insertion of a short branched linker. Because of this direct connection, the synthetic steps for the preparation of the GDN analogs have been reduced by two compared to that for GDN preparation.

The second and third sets (*i.e.*, SPSs and SPS-Ls) commonly have a penta-saccharide head group comprising one glucose core and four peripheral glucose units, attached to the four different lipophilic groups, but they are different from each other with respect to the head-to-tail connection mode (Scheme 2); direct linkage or a propylene spacer was used for connection in the SPSs or SPS-Ls, respectively.²¹ As the lipophilic groups, four structurally related steroids (cholestanol, cholesterol, sitosterol and diosgenin) were used to generate SPS-1/1L, SPS-2/2L, SPS-3/3L and SPS-4/4L, respectively. Cholestanol and diosgenin are the saturated and spiroketal versions of cholesterol, respectively, while sitosterol contains a β -ethyl branch in the alkyl region of cholesterol (C24 position). Thus, these lipophilic groups are structurally closed to each other yet differ from each other in terms of ring planarity, hydrophobic density of the alkyl region, or hydrophobicity of the lipophilic group. These structural variations allowed us to prepare a class of detergent molecules with different hydrophile-lipophile balance (HLB), known to be crucial for membrane protein stability.^{28,29} The SPSs and SPS-Ls were invented in order to 1) investigate the effects of the penta-saccharide head group in combination with the steroid architecture on membrane protein stability,²¹ and 2) also allowed us to introduce additional variability in the lipophilic group to include other steroid groups such as cholestanol and cholesterol. We hypothesized that, because of favorable efficacy of the pentasaccharide head group and increased hydrophobicity of the lipophilic group,²¹ these SPSs/SPS-Ls could more effectively interact with membrane protein surfaces, thereby resulting in enhanced protein stability.

The GDN variants were synthesized using straightforward synthetic pathways (see ESI†). The two hydroxyl groups used to attach the maltoside groups were derived from a double bond (C2-C3 or C5-C6) of the steroid unit. Specifically, C2- and C3-hydroxyl groups were produced from the double bond between C2 and C3 *via* epoxidation followed by epoxide ring opening by an acetate ion (GDN-1) or *via* osmium tetroxide-catalyzed *syn*-dihydroxylation (GDN-2) (Scheme 3). The C2-C3 double bond was obtained from ticoagenin, a saturated version of diosgenin, *via* solid phase-assisted elimination reaction. The SPSs were prepared using a protocol comprising four synthetic steps (*i.e.*, two repeated glycosylation and deprotection reactions) while the synthesis of the SPS-Ls required two

additional steps to insert a propylene spacer between the head and lipophilic groups (Scheme 3). Glycosylation was carried out stereo-specifically using benzoyl protected maltosylbromide/glucosylbromide as the glycosyl donor. Because of the presence of the carbonyl group in close proximity to an anomeric carbon (C1'), the acyloxonium ion forms as the main intermediate *via* neighbouring group participation (NGP), which preferentially produces a β -anomeric product. As expected, two maltose groups were connected to the diosgenin lipophilic group *via* β -glycosidic bonds, supported by the ^1H NMR spectra of the individual GDNs (GDN-1/2/3). The ^1H NMR spectra measured in CD_3OD solvent shows two doublet peaks at 4.30 and 4.35 ppm for GDN-1 and at 4.32 and 4.49 ppm for GDN-3, with a vicinal coupling constant (J) of 8.0 Hz (Figure S2). The chemical shift (δ) and coupling constant observed here are typical for β -anomeric protons. As for GDN-2, the ^1H NMR spectrum was measured in $\text{DMSO}-d_6$ due to a solubility issue in CD_3OD and did not give a clear indication of the stereochemistry of the glycosidic bonds because of peak overlap (Figure S2). GDN-1 with both α and β -glycosidic bonds showed peaks corresponding to the anomeric carbons at around 103 ppm in the ^{13}C NMR spectrum (Figure S3). The same β -stereochemistry was observed for all the glycosidic bonds of the SPSs except for one of the four peripheral glycosidic bonds (*i.e.*, C2'-O, C3'-O, C4'-O, or C6'-O) that turned out to be an α -linkage. For example, in the case of SPS-2, we observed five doublet peaks in the range of 4.3 to 5.3 ppm in the ^1H NMR spectrum, corresponding to the five anomeric protons (H_a) (Figure S4, *top*).

The four peaks appearing at 4.33, 4.54, 4.64, and 4.98 ppm with $J = 8.0$ Hz correspond to β -anomeric protons while the peak at 5.20 ppm with $J = 4.0$ Hz corresponds to the α -anomeric proton. For the syntheses of SPS-Ls, the tosylated steroid units were reacted with propane-1,3-diol. This reaction is known to proceed *via* an $\text{S}_{\text{N}}1$ mechanism, resulting in retention of the stereochemistry at the C3 of the steroid units.³⁰ The β -stereochemistry of C3-OR group was supported by the ^1H NMR spectra of the reaction products, showing a single C3-H peak centered at 3.16 ppm as a multiplet (Figure S5). The multiplicity and chemical shift (δ) of this peak are nearly the same as those obtained for the corresponding peak of a related compound with the same β -stereochemistry.³¹ Notably, in the SPS-L preparations, we barely detected the α -glycosidic linkage in the glycosylated products, in contrast to the results obtained for the SPSs. This result indicates that the presence of the propylene spacer has a critical role in the stereo-chemical outcome of the glycosylation reaction. For example, SPS-2L gave an NMR spectrum showing five doublet peaks at 4.38, 4.50, 4.66, 4.73 and 4.94 ppm with $J = 8.0$ Hz, indicative of all β -glycosidic bonds (Figure 1b & S4 (*bottom*)). The corresponding anomeric carbons gave multiple ^{13}C NMR peaks in the range of 102.5 to 104.9 ppm (Figure S6). SPS-2L was further characterized in terms of through-space proton-proton interactions *via* the 2D NOESY experiment (Figure S7). This agent gave NOE correlation signals between proton (H_6) and proton (H_7). Due to the close proximity in space a strong NOE correlation signal was found between H_7 or H_8 and methyl protons (C_{19} position). Distinctive NOE correlation signals were additionally observed between the protons (H_8 and methyl protons of C_{18} position) as well as the protons (H_{15} and methyl protons of C_{18} position).

All the new agents except GDN-3 were highly water-soluble (>10%). GDN-3 showed less than 1% water-solubility and thus was not characterized further. The limited water-solubility

observed for GDN-3 implies that a well-defined segregation between the hydrophilic and lipophilic groups is necessary for good water-solubility, a prerequisite to utility in membrane protein research. Aggregation behaviours of the GDNs and SPSs/SPS-Ls were characterized in terms of critical micelle concentrations (CMCs) and hydrodynamic radii (R_h) of their micelles. Detergent CMCs were measured by diphenylhexatriene (DPH) encapsulation,³² while the size and homogeneity of detergent micelles were estimated *via* dynamic light scattering (DLS) experiments. The summarized data for the new agents, a conventional detergent (DDM) and the original GDN are presented in Table 1. The CMCs of GDN-1 and GDN-2 were ~330 and ~270 μM , respectively, a little higher than that of DDM (170 μM), but substantially higher than that of GDN (~18 μM). In contrast, all SPSs and SPS-Ls except SPS-4/4L gave CMC values ranging from ~0.8 to ~3 μM , much lower than both the GDNs and DDM. A cholesterol-bearing detergent gave a little smaller CMC value than a cholesterol-bearing detergent (e.g., SPS-1 (~2 μM) *vs* SPS-2 (~3 μM). The presence of the ethyl branch in the lipophilic group or of the propylene spacer tend to lower detergent CMCs, as exemplified with SPS-3 (~2 μM) *vs* SPS-2 (~3 μM) or SPS-1L (~1 μM) *vs* SPS-1 (~2 μM). These decreased CMC values are likely due to the increased hydrophobicity of the detergent lipophilic group. SPS-4 and SPS-4L with diosgenin lipophilic group were more or less comparable to DDM and GDN variants in terms of CMC values, indicating that the presence of the ketal group in the terminal region of the lipophilic group significantly decreases detergent tendency to self-assemble presumably due to its relative high polarity. The sizes of micelles formed by the new GDNs (GDN-1 and GDN-2 (3.2 nm)) were smaller than that of GDN (3.9 nm) (Figure S8a). The SPSs except SPS-4 (3.1 nm) formed a little larger micelles (3.4 ~ 3.5 nm) than the GDNs and were comparable to DDM (3.4 nm) in this regard (Figure S8b). Interestingly, micelles were enlarged by addition of the propylene spacer between the individual steroidal units and the penta-saccharide head group (the SPSs *vs* SPS-Ls) (Figure S8c). For instance, SPS-2 micelles were estimated to have R_h of 3.4 nm, while SPS-2L with the propylene spacer formed micelles with R_h of 4.9 nm. Among the SPSs and SPS-Ls, SPS-4 and SPS-4L with the diosgenin lipophilic group formed the smallest micelles (3.1 and 3.9 nm, respectively). Detergent micelle size is heavily influenced by molecular geometry, estimated by the volume ratio of detergent head and tail groups.³³ The small micelles detected for the SPSs and SPS-Ls, despite bearing large steroidal units, are likely due to the presence of the bulky penta-saccharide head group. Detergent micelles were further analyzed in terms of size distribution of the micellar populations. The number- and volume-weighted DLS profiles of SPS-1L showed a single set of micellar populations, strongly indicating that the micelles formed by this agent are highly homogeneous (Figure 2a). Large aggregates appeared in the intensity-weighted DLS profile of this agent probably due to the extreme sensitivity of scattered light intensity to a large particle; scattered light intensity is known to be proportional to R^6 where R represents micelle size (Figure 2a).³⁴ Similar DLS profiles were observed for the other detergents (GDNs, SPSs and SPS-Ls) (Figures S8, S9 and S10). Detergent micelles were further investigated by transmission emission microscopy (TEM) analysis. As shown in Figure 2b, SPS-1L tends to self-assemble into spherical micelles in aqueous solution, with an estimated average diameter of 8.3 nm, consistent with that obtained from the DLS experiment ($D_h = 8.2$ nm) (Figure 2a). The presence of large aggregates in the image may be an artifact resulting from the sample preparation conditions. Wet and dry samples were used for DLS measurement and TEM

imaging, respectively. Of note, detergent micelles formed by these steroidal amphiphiles were stable enough to give clear solutions for more than a month at room temperature.

Detergent evaluation with multiple membrane proteins

To evaluate the potential of new detergents as biochemical tools for membrane protein study, these agents were first tested with the bacterial leucine transporter (LeuT) from *Aquifex aeolicus*.^{35,36} The transporter was first purified using 0.1% DDM and the individual novel agents were then introduced into sample solutions by diluting the DDM-purified LeuT into a buffer solution containing the respective detergent. Final detergent concentrations were CMC+0.04 wt%. DDM and GDN were used as positive controls. Protein stability was assessed by measuring the ability of the transporter to bind a radio-labeled substrate ($[^3\text{H}]$ -leucine (Leu)), termed transporter activity here, *via* scintillation proximity assay (SPA).³⁷ LeuT stability was monitored at regular intervals during a 12/13-day incubation at room temperature. Consistent with previous results,¹⁷ the transporter solubilized in GDN showed initial activity as high as for the protein in DDM and this high activity was maintained over the course of the 13-day incubation (Figure 3a). However, all new GDNs and SPSs yielded initial transporter activity substantially lower than DDM and were only comparable to this conventional detergent at retaining the initial transporter activity. Protein stability was substantially enhanced when the transporter was solubilized in each SPS-L, indicative of a favorable role of the flexible propylene linker in detergent efficacy. The transporters solubilized in the individual SPS-Ls reached enhanced activity compared to the transporters in the SPS counterparts after a 2-day incubation, but this protein activity was still a little lower than that of DDM-solubilized transporter (Figure 3b). Encouragingly, transporter activity in the SPS-Ls was retained for longer than that in DDM, as observed for GDN. Overall, these linker-bearing detergents were more or less comparable to DDM, with the best performance observed for SPS-1L. Interestingly, the SPSs or the SPS-Ls gave minor differences in transporter activity despite the fact that their hydrophobic groups (cholestanol, cholesterol, sitosterol and diosgenin) differ substantially from each other.

Next, these novel agents were further evaluated with a G protein-coupled receptor (GPCR),³⁸ the human β_2 -adrenergic receptor ($\beta_2\text{AR}$). For this experiment, DDM-purified receptor was first obtained by protein extraction from the membrane, followed by purification in 0.1% DDM. DDM was then exchanged into the individual novel agent (GDNs, SPSs, and SPS-Ls) by diluting the DDM-purified receptor into each detergent-containing buffer solution. At a final detergent concentration of CMC+0.2 wt%, protein stability was assessed by measuring the ability of the receptor to bind the radio-labeled antagonist ($[^3\text{H}]$ -dihydroalprenolol (DHA)).³⁹ When $\beta_2\text{AR}$ stability was measured 30-min after detergent exchange, all the new agents except SPS-4 were comparable to or better than DDM (Figure S11). Based on this preliminary result, the new GDNs (GDN-1/2), SPSs (SPS-1/2/3), and SPS-Ls (SPS-1L/2L/3L/4L) were selected for further evaluation, along with two positive controls (DDM and GDN). The ligand binding of the receptor was monitored at regular intervals during a 5-day incubation at room temperature. The DDM-solubilized receptor showed high initial activity (i.e., ligand binding ability), but this activity dropped rapidly with time (Figure 4). The two GDNs (GDN-1 and GDN-2) were similar to DDM in terms of initial receptor activity, but these GDNs, particularly GDN-1, were better than DDM in

preserving receptor activity long term (Figure 4a). GDN-1 efficacy was, at least, comparable to that of combined DDM and cholesteryl hemisuccinate (CHS) (DDM+CHS). CHS is known to stabilize many GPCRs.²⁶ The SPSs and SPS-Ls showed high initial receptor activity, but these two set of detergents behaved differently in retaining initial receptor activity; β_2 AR in the tested SPSs lost activity faster than DDM, while the receptor in the respective SPS-L showed a slower decrease in activity compared to DDM (Figure 4b & 4c). SPS-1L was most effective in this regard, followed by SPS-3L and SPS-2L. The best new agent (i.e., SPS-1L) appeared to be even better than GDN as receptor activity in this agent was higher than that in GDN over the total incubation period. This result implies that SPS-1L holds significant potential for GPCR structural study.

Encouraged by the results of the new agents with LeuT and β_2 AR, we next turned to the melibiose permease of *Salmonella typhimurium* (MelB_{St})⁴⁰ to further evaluate the new agents in terms of membrane protein solubilisation as well as stabilization. In this assay, the individual novel agents were directly used for protein extraction without introducing a conventional detergent (DDM), thereby giving results free of any residual DDM in the sample solutions. In order to investigate protein extraction efficiency, *E. coli* membranes containing MelB_{St} were treated with 1.5 wt% DDM, or the individual steroid-based detergents (GDNs, SPSs, and SPS-Ls), and incubated at 23 °C for 90 min. Following ultracentrifugation, the amount of soluble MelB_{St} was analyzed by SDS-PAGE and subsequent immunoblotting. DDM quantitatively yielded soluble MelB_{St} under the conditions while all the GDNs (GDN, GDN-1, and GDN-2) gave extraction efficiencies of 70–80% soluble transporter (Figure S12). This result indicates that these GDN variants are less efficient than DDM at extracting MelB_{St} from the membrane. Interestingly, the SPSs and SPS-Ls tended to show increased solubilisation efficiencies compared to the GDNs, resulting in efficiency of SPS-3/SPS-4L almost comparable to DDM. The superiority of the new agents compared to the conventional detergent (DDM) was detected in MelB_{St} thermo-stability. MelB_{St} thermo-stability in the individual detergents was assessed by further incubating the 23 °C-transporter extracts at three different temperatures (65, 70 and 75 °C) for an additional 90 mins. The amounts of soluble MelB_{St} in these thermally treated samples were estimated by the same protocol described above. Consistent with previous results,²³ DDM completely failed to maintain MelB_{St} in a soluble state at 65 °C (Figure 5a), suggesting that all MelB_{St} in this detergent denatured or aggregated in the course of the thermal treatment. The GDNs (GDN-1 and GDN-2), similar to GDN, retained about 25% soluble MelB_{St} under this temperature, indicating that the newly developed GDNs are better than DDM at retaining MelB_{St} solubility. Markedly enhanced detergent efficacy was obtained with the SPSs and SPS-Ls. At 65 °C, all SPSs and SPS-Ls except SPS-4/4L showed more than 85% MelB_{St} solubility, with a better performance observed for the SPS-Ls than the SPSs (Figure 5a). SPS-4 and SPS-4L were more or less comparable to the GDNs in this regard, which is likely due to the fact that these two agents and GDN share the diosgenin lipophilic group. This result implies that the presence of spiroketal moiety in the lipophilic group is suboptimal for detergent efficacy for MelB thermo-stability. The difference in detergent efficacy became more obvious when incubation temperature was further elevated to 70 and 75 °C. The individual SPSs and SPS-Ls except SPS-4/4L still retained significant amounts of soluble MelB_{St} even at these high temperatures, while only

very small amounts of soluble MelB_{St} were detected for the GDNs and SPS-4/4L (Figure 5a). Of the SPSs and SPS-Ls, SPS-2L and SPS-3L were the best, as these were capable of retaining quantitative amounts of soluble MelB_{St} even at 75 °C. It is noteworthy that most novel detergents developed to date have failed to yield noticeable amounts of soluble MelB_{St} even at 65 °C under similar conditions.^{15,24,41–44} Therefore, the current results indicate that the SPSs (SPS-1/2/3) and SPS-Ls (SPS-1L/2L/3L) are remarkably effective at maintaining the transporter in a soluble state. The best agents of the SPSs and SPS-Ls (SPS-3 and SPS-3L, respectively) were selected for analysis of MelB functionality. The functionality of MelB_{St} was estimated using two galactoside derivatives (melibiose and 2'-(*N*-dansyl)aminoalkyl-1-thio- β -D-galactopyranoside (D²G)).⁴⁵ Functional transporter strongly binds to the fluorescent D²G ligand, giving rise to high fluorescence emission intensity due to the occurrence of Förster resonance energy transfer (FRET) from tryptophan (Trp) in the MelB substrate binding site to the fluorescent ligand (D²G). This high fluorescence intensity is reversed by addition of excess melibiose (substrate) due to D²G-melibiose exchange in the binding site. Thus, the functional state of MelB_{St} can be assessed by monitoring changes in fluorescence emission intensity in the course of successive additions of D²G and melibiose. DDM-solubilized MelB_{St} gave rise to an increase in the fluorescence intensity upon addition of D²G which was reduced upon addition of melibiose (Figure 5b). When we used a less stable MelB homologue, MelB from *Escherichia coli* (MelB_{Ec}), however, this conventional detergent failed to produce a functional transporter;⁴⁵ no response was observed in fluorescence intensity upon addition of melibiose. In contrast, both MelB_{St} and MelB_{Ec} solubilized in the selected new detergents (SPS-3 or SPS-3L) gave fluorescence signals upon addition of D²G which was reduced upon addition of melibiose, indicating that these novel detergents were superior to DDM at maintaining MelB in a functional state.

Several studies have reported a judicious detergent choice based on individual detergent suitability for membrane protein manipulation.^{46–49} However, conventional detergents remain inadequate for the structural and functional studies of many membrane proteins.⁴⁹ In this context, diosgenin and other steroid-based groups such as cholestanol, cholesterol and sitosterol offer unique platforms for the development of novel detergents because of their rigid and planar architecture, along with high hydrophobic density. These structural features largely deviate from flexible alkyl chains used as the lipophilic group in typical conventional detergents. Thus, steroid-based novel agents possess detergent properties distinctive from most conventional detergents. However, there has been no extensive detergent study of steroid-based amphiphiles to date. In this regard, the novel steroidal amphiphiles (two GDNs, four SPSs and four SPS-Ls) introduced here displayed favourable behaviours toward membrane protein stability. For instance, SPS-1L outperformed both DDM and GDN in terms of long-term stability of β_2 AR. The favourable property of these rigid hydrophobic group-bearing agents was also seen with MelB_{St}. The thermo-stability of this transporter achieved by the use of the novel agents, particularly the SPS-Ls, was unprecedented. Three SPS-Ls (SPS-1L/2L/3L) increased thermo-stability of the transporter by ~30 °C compared to a gold-standard conventional detergent (DDM) (45 °C vs 75 °C), one of the largest differences in protein thermo-stability between a novel detergent and DDM. This MelB_{St} result clearly illustrates a distinctive advantage of these steroid-based detergents over conventional detergents. It is notable that these SPS-Ls were even superior to the previous

GDN, popularly used in membrane protein research.²⁷ However, the new agents were less effective overall with LeuT. Although the best agent for this prokaryotic transporter, SPS-1L, was a little inferior to GDN, this agent was comparable to DDM in retaining long-term stability, suggesting that this steroidal agent could be used as an alternative to DDM even for this less compatible protein. That detergent efficacy is dependent on the target protein as observed here is common, as membrane proteins are highly variable in 3D structures and properties.^{46,50}

GDN-1 and GDN-2 are stereoisomers of each other, with the relative orientations of the two maltoside groups different (*trans* and *cis*, respectively). With this isomeric variation, we found only a marginal difference in detergent efficacy for all three tested membrane proteins (LeuT, β_2 AR and MelB_{St}), indicating that the relative direction of the maltoside head groups is of little importance in protein stability. However, significant differences were observed in detergent efficacy between two sets of penta-saccharide-based detergent (SPSs and SPS-Ls), with the SPS-Ls better than the SPS equivalents for all tested membrane proteins. The SPSs and their SPS-L counterparts (e.g., SPS-1 and SPS-1L) have exactly the same lipophilic and head groups (a steroid unit and a penta-saccharide, respectively), but differ from each other in terms of the absence/presence of the propylene spacer at the hydrophilic-lipophilic interface. The presence of the alkyl spacer in the SPS-Ls is likely to increase detergent flexibility relative to the SPSs, which is mainly responsible for their enhanced efficacy for protein stability. As the steroid unit and penta-saccharide head group are both very rigid, the SPS molecules with no alkyl spacer may be less able to adopt conformations optimal for favorable detergent-detergent or detergent-protein interactions when associated with membrane proteins. It is notable that the lipophilic groups of these steroidal detergents have a planar architecture with large hydrophobic surfaces. Thus, *via* large London-dispersion forces, these lipophilic groups could strongly interact with the neighboring steroidal groups in the micellar environment. The SPS-Ls with more flexible architecture than the SPSs likely attain such strong hydrophobic interactions between the lipophilic groups, thereby contributing to their enhanced efficacy for protein stabilization. Based on this discussion, a structural modification of this spacer (e.g., variation in the spacer length) may enable us to further optimize these detergents for membrane protein stability.

A substantial variation in detergent efficacy was also observed by introduction of a small change in the structure of the lipophilic group, depending on the target membrane protein. As for LeuT stability, all SPSs behaved similarly as did all SPS-Ls, but substantial variations amongst each set of the detergents (SPSs or SPS-Ls) were detected in detergent evaluation with MelB_{St} and β_2 AR. It is particularly interesting to see a rather large efficacy difference between SPS-1L and SPS-2L. These agents only differ from each other in terms of the presence of double bond located between C5 and C6, but, despite this small structural difference, SPS-1L was superior to SPS-2L in stabilizing β_2 AR while an opposite trend was observed for MelB_{St} stability. The absence of the C5-C6 double bond seems particularly favorable for β_2 AR stability. Overall, detergent efficacy tends to be strongly affected by a small change in detergent structure, illustrating a key challenge in the development of novel detergents for membrane protein study. Multiple detergent properties such as HLB, alkyl chain length and molecular flexibility need to be considered in detergent design and only a narrow range of those properties are allowed for an effective novel detergent.

Conclusions

With variations in relative direction of two maltoside head groups (*cis* or *trans*), in lipophilic group structure (cholestanol, cholesterol, sitosterol, or diosgenin), and in detergent flexibility *via* spacer use, the three sets of steroid-bearing detergents (GDNs, SPSs and SPS-Ls) were designed, prepared and evaluated as chemical tools for membrane protein solubilization and stabilization. In general, amongst these sets of new agents, the SPS-Ls displayed most favorable behaviors for membrane protein stabilization. Some SPS-Ls, as exemplified with SPS-1L for LeuT and β_2 AR, or SPS-2L/3L for MelB_{St}, were superior to DDM in retaining protein stability. Overall performance of these SPS-Ls (SPS-1L for β_2 AR and SPS-2L/3L for MelB_{St}) was even superior to the original GDN. Furthermore, these new agents are more accessible than GDN because they can be prepared using a reduced synthetic protocol and high synthetic yield. This together with their efficacy for membrane protein stabilization, means that these SPS-Ls have clear potential as biochemical research tools. In addition, the role of the alkyl spacer in detergent efficacy for protein stabilization suggested here should provide a detergent structure-efficacy relationship useful for future detergent design and development.

Experimental Section

Synthesis and characterization of novel amphiphiles, and membrane protein stability assays: Experimental details can be found in the Supporting Information.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(a) Chemical structure of SPS-2L illustrating the anomeric protons (H_a or H_{a'}), their couplings with the neighboring protons (H in blue) and (b) partial ¹H NMR spectrum of this detergent focusing on the anomeric region (see Figure S4[†] for the full range of ¹H NMR spectrum). The spectrum shows five doublets at 4.38, 4.50, 4.66, 4.73 and 4.94 ppm, with the coupling constant (3J_{aa}) of 8.0 Hz, typical peak characteristics for β-anomeric proton (H_a/H_{a'}).

Figure 2.

(a) DLS profile and (b) TEM image of SPS-1L. The DLS data and TEM image were obtained at a detergent concentration of 1.0 wt%. Detergent sample for TEM analysis was visualized by staining with 2% phosphotungstic acid. Number- and volume-weighted DLS profiles showed a single set of micellar populations while the intensity-weighted profile gave two sets of populations. The large micelles appearing in TEM image may be an artifact resulting from dried sampling conditions. Abbreviations: DLS, dynamic light scattering; TEM, transmission electron microscopy.

Figure 3.

Long-term stability of LeuT in the presence of individual detergents. Detergents included GDNs (GDN-1 and GDN-2), SPSs (SPS-1, SPS-2, SPS-3, and SPS-4), and SPS-Ls (SPS-1L, SPS-2L, SPS-3L and SPS-4L) used at CMC+0.04 wt%. DDM and GDN were used as positive controls. Protein stability was assessed by measuring the substrate binding ability of the transporter using [³H]-leucine (Leu). Transporter stability was monitored regularly during a 12/13-day incubation at room temperature *via* scintillation proximity assay (SPA). Error bars, SEM, *n* = 3.

Figure 4.

Long-term stability of β_2 AR solubilized in (a) GDNs (GDN-1, and GDN-2), (b) SPSs (SPS-1, SPS-2, and SPS-3) and (c) SPS-Ls (SPS-1L, SPS-2L, SPS-3L, and SPS-4L). DDM, DDM combined with cholesteryl hemisuccinate (DDM+CHS), and GDN were used as positive controls. Detergent exchange was carried out by diluting DDM-purified β_2 AR into the individual detergent-containing buffer solutions to give a final detergent concentration of CMC+0.2 wt%. Protein stability was assessed by measuring the ability of the receptor to bind the radio-labeled antagonist ([³H]-dihydroalprenolol (DHA)) over the course of a 5-day incubation at room temperature. Error bars, SEM, $n = 3$.

Figure 5.

(a) Thermo-stability of MelB_{St} solubilized in novel steroid-based amphiphiles (GDNs, SPSs, and SPS-Ls). DDM and GDN were used as positive controls. MelB_{St} was extracted with a detergent at 23 °C, and after ultracentrifugation, the supernatant was further incubated for another 90 mins at an elevated temperature (65, 70 or 75 °C). These thermally treated samples were analyzed by SDS-PAGE and Western blotting (top panel) after ultracentrifugation again. Histograms (bottom panel) show the amounts of soluble MelB_{St} obtained from Western blots. The amounts of soluble transporter in the individual conditions are represented as percentages of soluble MelB_{St} present in the individual protein extracts at 23 °C (a, Ext). Error bars, SEM, n = 3. (b) MelB functional assay. Right-side-out (RSO) membrane vesicles containing MelB_{St} or MelB_{Ec} were treated with DDM, SPS-3 and SPS-3L and the resulting MelB extracts, after ultracentrifugation, were then subjected to functional assay (i.e., melibiose reversal of FRET from Trp to dansyl-2-galactoside (D²G)).

Fluorescence emission intensity was monitored over the course of additions of D²G and an excess amount of melibiose at the 1-min and 2-min time points, respectively (orange line). Water instead of melibiose was added to the assay solutions to obtain control data (blue line).

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Scheme 1.

Chemical structures (left) and Newman projections (right) of GDN analogs (GDN-1, GDN-2 and GDN-3). Two maltosides were directly introduced to the diosgenin lipophilic group using the C2 and C3 hydroxyl groups for GDN-1 and GDN-2, or using the C3 and C6 hydroxyl groups for GDN-3. Consequently, the two maltoside head groups are next to each other in GDN-1/2, while these head groups are three bonds away in GDN-3. The relative orientation along with dihedral angle and position of the two maltoside groups are indicated in the individual Newman projections.

Scheme 2.

Chemical structures of the steroid-based penta-saccharide amphiphiles (SPSs). A penta-saccharide head group was attached onto four different steroid-based hydrophobic groups: cholestanol (SPS-1 and SPS-1L), cholesterol (SPS-2 and SPS-2L), sitosterol (SPS-3 and SPS-3L), and diosgenin (SPS-4 and SPS-4L). The head and tail groups were connected either directly (SPSs) or via a propylene linker (SPS-Ls). The four consecutive conformationally-locked rings are designated A, B, C, and D, respectively, in the chemical structure of SPS-1/1L.

Scheme 3.

Synthetic scheme for the preparation of GDN-1 (*top*), SPS-1 (*middle*) or SPS-1L (*bottom*). GDN-1 was prepared in six synthetic steps starting from diosgenin while SPS-1 and SPS-1L were prepared in four and six synthetic steps, respectively, using 3β -cholestanol as the starting material. Diosgenin diol (compound **A**) and monoglucosylated cholesterol derivatives (compounds **B** and **C**) were used as glycosyl acceptors in the selective β -glycosylation.

Table 1.

Molecular weights (MWs), critical micelle concentrations (CMCs) of the steroid-based amphiphiles (GDNs, SPSs and SPS-Ls) and hydrodynamic radii (R_h ; $n = 5$) of their micelles in water at room temperature.

Detergent	MW^a	CMC (μM)	CMC (wt%)	R_h (nm) ^b
GDN-1	1081.2	~330	~0.036	3.2±0.0
GDN-2	1081.2	~270	~0.029	3.2±0.1
SPS-1	1199.4	~2	~0.0002	3.4±0.1
SPS-2	1197.4	~3	~0.0003	3.4±0.2
SPS-3	1225.4	~2	~0.0002	3.5±0.1
SPS-4	1225.3	~150	~0.018	3.1±0.2
SPS-1L	1257.5	~1	~0.0001	4.1±0.1
SPS-2L	1255.5	~3	~0.0004	4.9±0.1
SPS-3L	1283.5	~0.8	~0.0001	4.9±0.1
SPS-4L	1283.4	~200	~0.026	3.9±0.0
GDN	1165.3	~18	~0.0021	3.9±0.1
DDM	510.1	~0.17	~0.0087	3.4±0.0

^[a] Molecular weight of detergents.

^[b] Hydrodynamic radius of detergents measured at 1.0 wt% by dynamic light scattering.