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Glyco-Steroidal Amphiphiles (GSAs) for Membrane Protein Structural Study

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Integral membrane proteins pose considerable challenges to high resolution structural analysis. Maintaining membrane proteins in their native state during protein isolation is essential for structural study of these bio-macromolecules. Detergents are the most commonly used amphiphilic compounds for stabilizing membrane proteins in solution outside a lipid bilayer. We previously introduced a glyco-diosgenin (GDN) detergent that was shown to be highly effective at stabilizing a wide range of membrane proteins. This steroidal detergent has additionally gained attention due to its compatibility with membrane protein structure study *via* cryo-EM. However, synthetic inconvenience limits widespread use of GDN in membrane protein study. To improve its synthetic accessibility and to further enhance detergent efficacy for protein stabilization, we designed a new class of glyco-steroid-based detergents using three steroid units: cholestanol, cholesterol and diosgenin. These new detergents were efficiently prepared and showed marked efficacy for protein stabilization in evaluation with a few model membrane proteins including two G protein-coupled receptors. Some new agents were not only superior to a gold standard detergent, DDM (*n*-dodecyl-β-D-maltoside), but were also more effective than the original GDN at preserving protein integrity long term. These agents represent valuable alternatives to GDN, and are likely to facilitate structural determination of challenging membrane proteins.

Introduction

Integral membrane proteins account for around a quarter of the human proteome.[1] These proteins play an essential role in countless cellular functions and are involved in many diseases. Thus, it is not surprising that membrane proteins are targets of over 40% of FDA-approved drugs,[2,3] and that determination of their three-dimensional structures is of paramount importance. Structure determination of membrane proteins has historically been a technically challenging endeavour illustrated by the fact that membrane proteins comprise less than 3% of all structures deposited in the Protein Data Bank (PDB).[4] These challenges can arise at any point in the workflow, from gene expression to structural determination, including inadequate protein expression, poor extraction from the native lipid bilayers, and limited long-term stability over the course of sample preparation for the structure determination *via* either X-ray crystallography, single particle cryo-electron microscopy (cryo-EM) or nuclear magnetic resonance (NMR) spectroscopy.[5] Over the last decades, technological advances in expression systems, protein extraction and purification techniques, and structure determination methods have led to an ever-growing number of deposited membrane protein structures.[6–8] However, structural and functional studies of these proteins are still far more difficult than those of soluble proteins, mainly due to their instability in non-lipidic environments. The planar architecture of lipid bilayers is most suitable for protein stability because this arrangement exerts a strong lateral pressure on the membrane proteins. In addition, some membrane lipids, in addition to cholesterol, are specifically associated with membrane protein surfaces and have critical roles in protein function.[9–11] For downstream characterisation, these bio-macromolecules have to be extracted from the membranes and thus we need a membrane mimetic system that preserves the native structures of membrane proteins in non-native environments.

Structural characterizations of most membrane proteins have been achieved using detergents, amphipathic molecules with the ability to form a micellar sheath around the hydrophobic domains of the protein. In addition, detergent micelles are popularly used for membrane protein extraction and purification. Alkyl glucosides (e.g., OG (*n*-octyl-β-D-glucoside)),

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maltosides (e.g., DM (*n*-decyl-β-D-maltoside), DDM (*n*-dodecyl-β-D-maltoside)), amine oxides (e.g., LDAO (lauryldimethylamine-*N*-oxide)) and oligo-oxyethylenes (e.g., tetraoxyethylene glycol monooctyl ether (C_8E_4)) are single alkyl-chained detergents useful for membrane protein structural study.[12] However, due to their canonical architecture comprising single head and tail groups, membrane proteins in these conventional detergents often undergo protein aggregation/denaturation.[13,14] Conventional detergents with a narrow scope of structures are limited in their ability to effectively stabilize a large number of membrane proteins with diverse structures. Substantial efforts have been devoted to the development of new membrane mimetics over the past two decades, resulting in the invention of various amphiphilic systems. Representatives include bicelles,[15] nanodiscs (NDs),[16] polymeric amphiphiles (e.g., amphipols (APols)[17] and styrene-maleic acid copolymers (SMAs))[18] and peptide-based detergents (e.g., lipopeptides and Salipro).[19–20] Small amphiphilic agents structurally different from classical detergents have also been developed, as exemplified by the neopentyl glycol amphiphiles (glucose-neopentyl glycols (GNGs), maltose-neopentyl glycols (MNGs) and neopentyl glycol-derived triglucosides (NDTs)),[21–23] rigid hydrophobic group-bearing detergents (chobimalt, glyco-diosgenin (GDN), terphenyl group-bearing maltosides (TPMs)),[24–26] rigid linker-bearing detergents (resorcinarene-based amphiphiles (RGAs), norbornane-based maltosides (NBMs), scyllo-inositol glycosides (SIGs), and cyclopentane-based maltosides (CPMs))[27–30] and facial amphiphiles (lithocholate-based facial amphiphiles (LFAs) and tandem facial amphiphiles (TFAs)).[31,32] New hydrophobic or hydrophilic groups have been utilized to design novel detergents as exemplified with the dendronic trimaltosides (DTMs) and vitamin E-based glucosides (VEGs) or penta-saccharide-bearing amphiphiles (PSEs) and oligoglycerol detergents (OGDs).[33–36] Some of these agents such as OGNG, LMNG and GDN have contributed to high resolution structural determinations of membrane proteins *via* cryo-EM or X-ray crystallographic methods.[37–39]

Of these novel detergents, GDN has gained attention as an agent particularly suited to membrane protein structural study. This detergent is a synthetic molecule with high homogeneity to digitonin, a steroidal saponin obtained from the purple foxglove plant *Digitalis purpurea*.[40–43] GDN and digitonin differ in terms of their head groups and the substitution pattern of the hydrophobic ring (Figure S1). This agent is also different from other structurally related detergents such as chobimalt and 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS).[24,44] Chobimalt has a linear tetra-saccharide head group, while CHAPS contains multiple hydroxyl groups facially projecting from the steroid scaffold, along with the zwitter-ionic head group. GDN has been increasingly used for membrane protein structural study since its invention. This steroidal detergent has contributed to the determination of more than 150 membrane protein structures over the past four years (2018 to 2021), representing ~12% of the total number of membrane protein structures reported over the same period.[45] The use of GDN in membrane protein structural study has increased recently; the number of protein structures obtained using this detergent has increased from 4 (2018) to 35 (2019) to 63 (2020). As of December 31, 2021, 68 membrane protein structures were reported that year where GDN was used for protein solubilisation, purification and/or structure determination.[45] Diverse classes of membrane proteins, including G protein-coupled receptors (GPCRs), channels, transporters, and enzymes, have been structurally characterized using GDN. Examples include the human cholesterol transporter NPC1 (PDB: 6W5S),[46] human pannexin 1 channel (PDB: 6WBF),[47] the glutamate transporter VGLUT2 (PDB: 6V4D),[48] human calcium homeostasis modulator ion channels CALHM4 and CALHM6 (PDB: 6YTK/6YTV),[49] mitochondrial respiratory super-complex (PDB: 6T15),[50] and the amino acid transporter $b^{0,+}$AT-rBAT (PDB: 6LID).[51] These successes indicate that the GDN scaffold is highly compatible with membrane protein structural studies. Despite the popularity of GDN in membrane protein studies, its preparation requires seven synthetic steps with an overall low yield (~35%). Thus, we have designed new detergents using the steroidal units (cholestanol, cholesterol and diosgenin) as the hydrophobic group, designated glyco-steroidal amphiphiles (GSAs) (Figure 1). These new agents were tested with multiple membrane proteins to evaluate their efficacy for protein solubilisation and stabilization.

Results and Discussion

Detergent structures and physical characterizations

The new detergents introduced in the current study share a branched dimaltoside head group with the previously reported GDN (Figure S1). However, the maltose groups of the previous GDN and the new detergents are connected to the hydrophobic groups *via* different linkers. The linker for GDN contains two primary alcohol groups while the linkers used for preparation of the new detergents have two different alcohol groups (primary and secondary). In addition, the new detergents vary in the distance between the head and tail groups, a difference attained by utilizing different lengths of linkers. The variation in head-to-tail distance, indicated in detergent designation by C1, C2 and C3, is necessary to find an optimal distance for protein stability. With regard to the hydrophobic groups, three structurally related steroids (cholestanol, cholesterol and diosgenin) were used to generate the three sets of new detergents: GCAs, GCEs and GDNs, respectively. Cholestanol is a saturated version of cholesterol, while diosgenin is a spiroketal-functionalized version of cholesterol. These lipophilic groups are structurally related, yet differ from each other in terms of ring planarity and hydrophobicity. These structural variations should facilitate identification of a detergent with optimal properties for membrane protein stabilization including micellar packing, protein-detergent interaction and hydrophilic–lipophilic balance (HLB).[52,53]

These novel agents were prepared using a synthetic protocol comprising five synthetic steps. Following tosylation of the alcohol group, each steroid unit (cholestanol, cholesterol or diosgenin) was reacted with a commercially available reagent

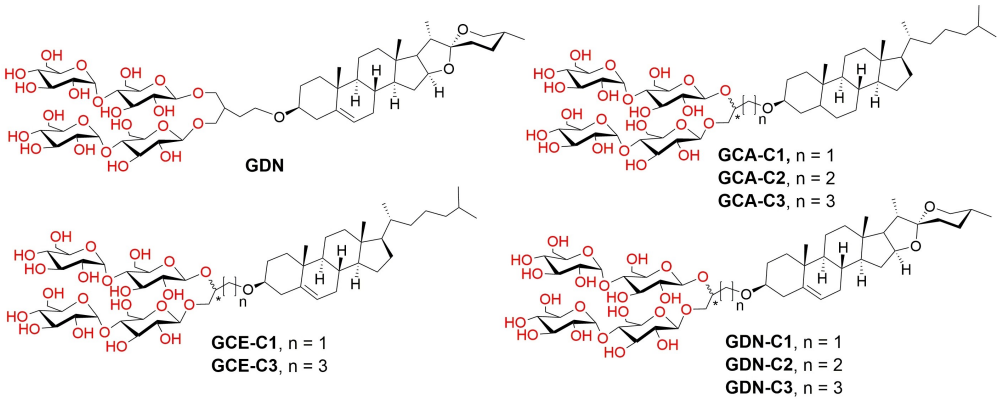
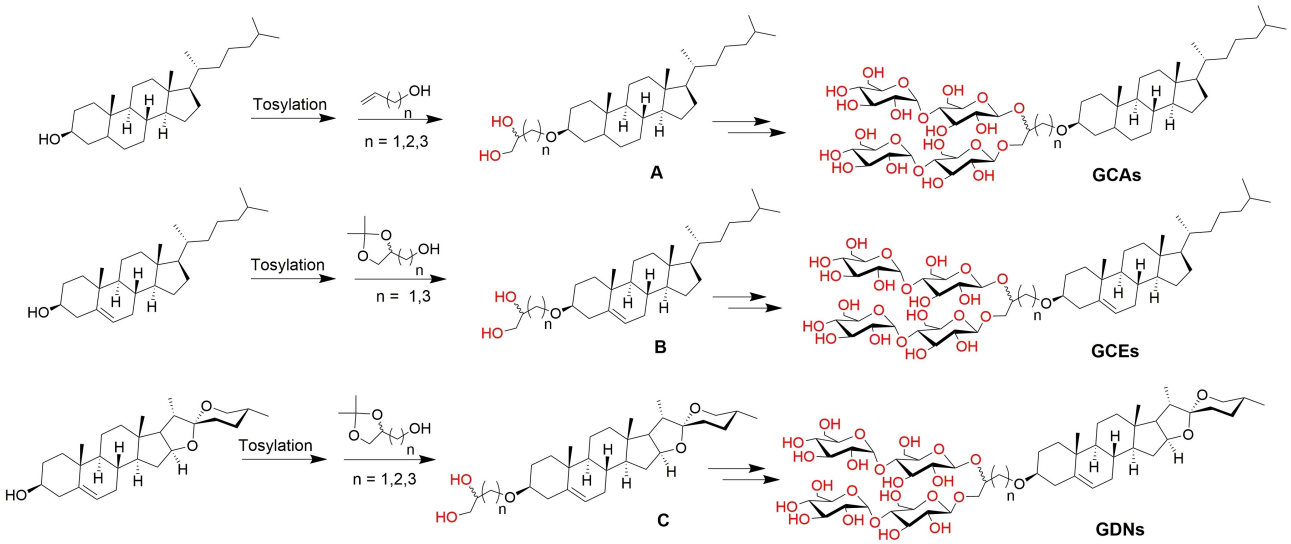


Figure 1. Chemical structures of previously reported GDN and newly prepared glyco-steroidal amphiphiles (GSAs). All GSAs share a branched dimaltoside hydrophilic group, but differ from each other in terms of the structures of their hydrophobic groups: cholestanol (GCA-C1, GCA-C2 and GCA-C3), cholesterol (GCE-C1, and GCE-C3) and diosgenin (GDN-C1, GDN-C2 and GDN-C3). The head and tail groups were connected using short linkers with different chain lengths. The chain length of the linker is indicated by C1, C2 or C3 in the detergent designation. The wavy line indicates the stereo-chemically undefined bond; the detergents are a mixture of two stereoisomers with *R* or *S* configuration on the asymmetric carbon (represented by the asterisk).

(double bond- or dioxolane-containing mono-ol derivative) (Scheme 1).[54,55] Next, the 1,2-diol-functionalized steroid derivatives (**A**, **B** and **C**) were prepared from dihydroxylation of the double bond (**A**) or acetonide deprotection of the dioxolane-protected glycerol linkers (**B** and **C**). The resulting compounds (**A**, **B** and **C**) were subjected to stereo-selective glycosylation, followed by global deprotection to yield the GCAs, GCEs and GDNs, respectively. The overall yields were in the range of 60 to 70%, significantly higher than that of GDN (~35%). The distance between the head and tail group systematically varied as a result of different chain lengths of double bond- or dioxolane-containing alcoholic linkers. Of note, GCE-C2 could not be prepared due to the unsuccessful coupling of the tosylated cholesterol with the dioxolane-protected glycerol derivative, for as yet unknown reasons. The dioxolane-protected glycerol derivatives were used as linkers to generate the 1,2-diol-functionalized steroids (**B** and **C**) due to the presence of the double bond in the cholesterol and diosgenin units. These double bonds were suspected to undergo cleavage under the conditions of OsO_4-catalyzed dihydroxylation. The diol-functionalized steroids (**A**, **B** and **C**) were isolated in a single chromatographic separation by combining the initial three synthetic steps together, adding to the synthetic convenience of the new detergents compared to GDN and other steroid-based detergents. The β-stereochemistry for the newly formed glycosidic bonds was confirmed by the individual ^{1}H spectra of the GSAs



Scheme 1. Synthetic scheme for preparation of the new glyco-steroidal amphiphiles (GCA-C1/C2/C3, GCE-C1/C3 and GDN-C1/C2/C3). Left: Three different steroidal moieties (cholestanol, cholesterol and diosgenin) were used as starting material. Two different kinds of linkers were introduced to the steroidal units for 1,2-diol functionalization: double bond-containing alcohol derivatives (cholestanol) and dioxolane-protected glycerol derivatives (cholesterol and diosgenin). Middle: The resulting diol-functionalized compounds (**A**, **B**, and **C**) were subjected to glycosylation and deprotection to yield the three sets of GSAs (GCAs, GCEs and GDNs). Right: The final products.

in CD_3OD (Figures S2–S4). The presence of multiple doublets in the range of 4.25 to 4.75 ppm is strong evidence for the presence of the β-glycosidic bonds. An α-anomeric proton typically produces an NMR peak around 5.15 ppm. The anomeric peaks corresponding to the protons attached to the anomeric carbons are rather complex because the maltose groups are connected to two different alcohol groups (primary and secondary). The presence of the stereo-chemically undefined carbon, as indicated by the wavy bond in the chemical structure, further adds to the complexity of the anomeric signals. As the dihydroxylation reaction of the double bond has limited stereo-selectivity and we used racemic mixtures of dioxolane-protected glycerol linkers, it is likely there is little batch-to-batch variation in detergent stereochemistry. The coupling constants of the anomeric peaks ($^3J = 4.0$ and 8.0 Hz for α- and β-anomeric protons, respectively) further support the successful synthesis of the target compounds.

All the new agents were highly soluble in water (> 10 % w/v) and the self-assemblies formed by these agents were stable as indicated by clear detergent solutions after one month storage at room temperature. Aggregation behaviours of the GSAs were investigated by measuring critical micelle concentrations (CMCs) and hydrodynamic radii (R_h) of the micelles in aqueous solution. A summary of the data for the GSAs along with DDM and the original GDN are presented in Table 1. All the detergents introduced here gave CMCs comparable to or lower than the original GDN (~ 18 μM). The diosgenin-derived detergents (GDN-C1/C2/C3) gave higher CMCs compared to the cholestanol- or cholesterol-based counterparts (*i.e.*, GCA-C1/C2/C3 or GCE-C1/C3). This is likely due to the presence of the polar spiroketal group at the terminal end of the hydrophobic group. Detergent CMC values tend to be inversely proportional to the chain length of the spacer between the head and tail groups, as exemplified by GCA-C1 (~ 4 μM) to GCA-C2 (~ 2 μM) to GCA-C3 (~ 0.8 μM). Micelles formed by the GSAs were comparable to or slightly larger than those formed by GDN (3.9 nm) (Table 1). The cholesterol-based detergents (GCEs) tend to form larger micelles than cholestanol- or diosgenin-based detergents, but the differences were relatively small. The similarity of the chemical structures of the GCAs, GCEs and GDNs explain the small differences in micelle size. A further analysis of the DLS data revealed that micelles formed by all new agents showed unimodal size distributions (Figures S5 & S6).

Table 1. Molecular weights (MWs), critical micelle concentrations (CMCs) of GSAs (GCAs, GCEs and GDNs) along with the original GDN and a single alkyl-chained detergent (DDM), and hydrodynamic radii (R_h) (mean ± S.D., $n = 5$) of their micelles.

Detergent	*M.W.*[a]	CMC (μM)	CMC (wt %)	R_h (nm)[b]
GCA-C1	1111.3	~ 4	~ 0.0004	3.8 ± 0.1
GCA-C2	1125.4	~ 2	~ 0.0002	4.0 ± 0.2
GCA-C3	1139.4	~ 0.8	~ 0.00009	5.7 ± 0.2
GCE-C1	1109.3	~ 2	~ 0.0002	4.3 ± 0.2
GCE-C3	1137.4	~ 0.7	~ 0.00008	6.5 ± 0.1
GDN-C1	1137.3	~ 20	~ 0.0023	3.9 ± 0.1
GDN-C2	1151.3	~ 10	~ 0.0014	3.9 ± 0.1
GDN-C3	1165.3	~ 4	~ 0.0005	4.2 ± 0.1
GDN	1165.3	~ 18[c]	~ 0.0021	3.9 ± 0.1
DDM	510.6	~ 170	~ 0.0087	3.4 ± 0.1

[a] Molecular weight of detergents. [b] Hydrodynamic radius of detergents measured at 1.0 wt % by dynamic light scattering. [c] Value reported in the literature.[25]

Detergent evaluation with membrane proteins

The GSAs were first evaluated with melibiose permease of *Salmonella typhimurium* ($MelB_{St}$).[56–58] $MelB_{St}$ was first produced in *E. coli* membranes and then extracted at 0 °C using each detergent at 1.5 wt % for 90 min, and then, without removal of the insoluble membrane fraction, the detergent extracts were further thermally treated at a higher temperature (45, 55, or 65 °C) for another 90 min. The amounts of soluble $MelB_{St}$ in each sample were analysed *via* Western blot and presented as percentages (%) of total transporter originally present in the untreated membranes. This two-step procedure allowed us to evaluate detergent efficiency/efficacy for membrane protein solubilisation and stabilization. The amount of soluble $MelB_{St}$ detected at 0 °C is a measure of the solubilisation efficiency of each detergent. The second thermal treatment of the detergent-solubilized protein accelerates protein denaturation/aggregation to varying degrees dependent on the stability of $MelB_{St}$ in a given detergent. Thus, the amounts of soluble $MelB_{St}$ detected at the elevated temperatures can be correlated with the detergent efficacy for protein stabilization. At 0 °C, DDM quantitatively yielded soluble $MelB_{St}$ under these conditions, whereas all new agents gave reasonable but lower amounts of soluble transporter (30 ~ 60 %) (Figure 2). This result indicates that these steroidal detergents are inferior to DDM at extracting $MelB_{St}$ from the membrane. Interestingly, the diosgenin-derived detergents (GDN, GDN-C1, GDN-C2, and GDN-C3) were generally more efficient than the cholestanol/cholesterol-derived agents (GCAs/GCEs) at extracting $MelB_{St}$ from the membrane. When the detergent extracts were incubated at 45 °C, the amounts of soluble $MelB_{St}$ dramatically increased in the case of all GSAs, giving yields of between 70 and 100 %. These improved efficiencies originate from further solubilisation of $MelB_{St}$ that had remained insoluble during protein extraction at 0 °C. The increased membrane dynamics and/or enhanced detergent solubility at the elevated temperature facilitated $MelB_{St}$ solubilisation. A clear differentiation in detergent efficacy between DDM and the new detergents was found when the temperature were further elevated to 55 °C. Use of DDM at this high temperature resulted in almost complete loss in soluble $MelB_{St}$, likely as the result of protein aggregation. In contrast, the use of the GSAs resulted in only a slight decrease in the amounts of soluble $MelB_{St}$ under the same conditions. All new detergents were comparable to original GDN in this regard. Even at a temperature of 65 °C, substantial amounts of soluble $MelB_{St}$ (30–70 %) were detected for all the individual new detergents, with GCA-C3 and GCE-C1 yielding the greatest amounts of soluble protein.

As GCE-C1 was the most effective overall at retaining $MelB_{St}$ solubility at 55 °C and GDN-C3 was the best of the GDN analogues (GDN-C1/C2/C3), these two agents (GCE-C1 and

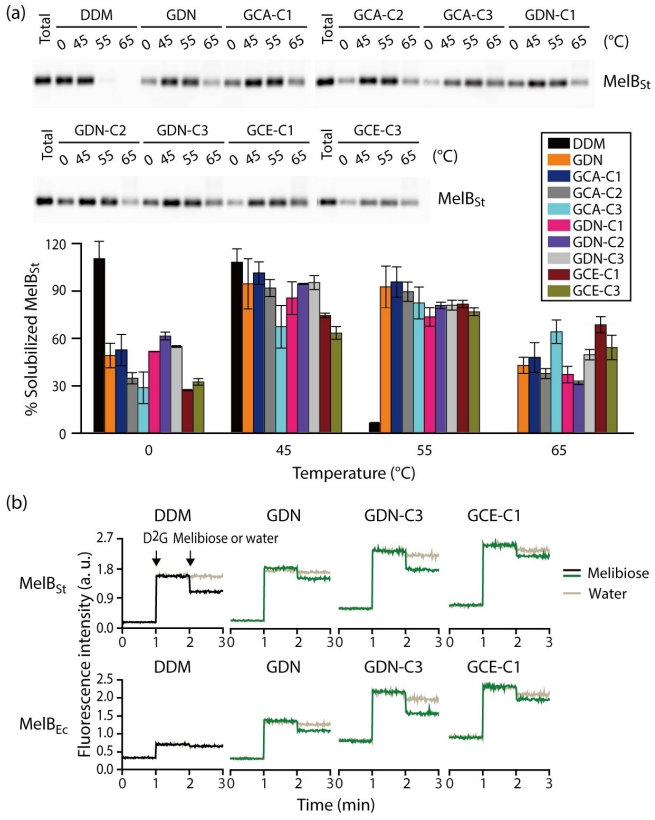


Figure 2. (a) Thermo-stability of $MelB_{St}$ in the new detergents (GCA-C1/C2/C3, GCE-C1/C3 or GDN-C1/C2/C3). DDM and GDN were used as controls. *E. coli* membranes containing $MelB_{St}$ were treated with the individual detergents at 0 °C for 90 min. (*top*) Detergent extracts were analysed by SDS-PAGE and Western blotting following ultracentrifugation, or further incubated at an elevated temperature (45, 55, or 65 °C) for another 90 min, followed by analysis using the same protocol. (*bottom*) The amounts of soluble $MelB_{St}$ are expressed as percentages of total $MelB_{St}$ present in the untreated membranes (histogram). Error bars, SEM, $n = 3$. (b) Galactoside binding of two MelB homologues in the selected detergents (GCE-C1 and GDN-C3). DDM and GDN were used as references. MelB function was assessed using FRET reversal based on a ligand-substrate exchange in the active site. Right-side-out (RSO) membrane vesicles containing $MelB_{St}$ or $MelB_{Ec}$ were solubilized with DDM, GDN, GCE-C1, or GDN-C3. Each detergent-solubilized MelB was treated with dansyl-2-galacotside (D^2G; 1-min) and excess melibiose (2-min) in a sequential manner. Variations in fluorescence intensity were monitored over the course of these additions. Control data was obtained by addition of water instead of melibiose. Melibiose and water additions are indicated in black (DDM)/green (a new detergent) and pale brown, respectively.

GDN-C3) were selected for further investigation of the functionality of detergent-extracted $MelB_{St}$. Starting with right-side out membrane vesicles,[56] $MelB_{St}$ solubilized in each detergent was ultracentrifuged and then was subjected to a FRET assay using Trp→dansyl-galactoside (2′-(*N*-dansyl)aminoalkyl-1-thio-β-D-galactopyranoside, D^2G).[58–60] Upon addition of D^2G, $MelB_{St}$ in a functional form exhibits an increased fluorescence intensity over time. A subsequent decrease caused by replacement of D^2G with non-fluorescent melibiose reflects the specific FRET from $MelB_{St}$ Trp residues to the dansyl moiety of bound D^2G in the sugar binding site. Notably, since the FRET intensity depends on factors including environment and protein conformation, the absolute FRET intensity (e.g., decrease by melibiose) does not correlate to binding affinity or protein stability. Smaller changes in FRET intensity do not mean a lower binding affinity, as demonstrated by isothermal titration calorimetry analysis.[57] Keeping this mind, the DDM-solubilized transporter showed a specific FRET signal, indicating transporter function (Figure 2b). However, a less stable homolog of $MelB_{St}$ in *E. coli*, $MelB_{EC}$, lost the sugar binding capability in DDM solution. Remarkably, the selected detergents (GCE-C1 and GDN-C3) along with GDN, were effective at preserving the functionality of both MelB homologues under the same conditions. Thus, the new detergents are effective at maintaining MelB in a soluble and functional form.

Next, we moved to bacterial leucine transporter (LeuT) from *Aquifex aeolicus*.[61,62] The transporter was first purified with 0.1% DDM, and the new agents were individually introduced into sample solutions *via* dilution. Final detergent and residual DDM concentrations were 0.04 and 0.03 wt%, respectively. Protein stability was assessed by measuring the ability of the transporter to bind a radiolabelled substrate ([^{3}H]-leucine (Leu)) through a scintillation proximity assay (SPA).[63] LeuT stability was monitored at regular intervals during a 13-day incubation at room temperature (Figure S7). The DDM-solubilized LeuT showed high Leu binding initially, but lost initial binding ability rapidly over time. Most of the new agents were superior to DDM at stabilizing the transporter long term, with the best performance observed for GDN, followed by GDN-C1, GDN-C3 and GCA-C1. Interestingly, GCA-C3 and GCE-C3 failed to stabilize the transporter, in contrast to the high efficacy of GDN-C3 with the same linker. This result indicates that the GDN scaffold is more suitable for LeuT stability compared to the cholestanol/cholesterol scaffold. This was further supported by the markedly enhanced efficacies of all new GDNs and the original GDN for stabilising LeuT compared to DDM. Interestingly, detergent efficacy for LeuT stabilization tended to decrease with increasing chain length of the linker. This result implies that detergents that are largely hydrophobic are suboptimal for LeuT stability. GCA-C1 and the GDNs are relatively hydrophilic due to the presence of a short linker and a hydrophilic spiroketal group, respectively, and are therefore effective at stabilizing LeuT. Based on the favourable effects of the GDNs and GCA-C1 on the stability of MelB and LeuT, it is likely that these agents represent alternatives to standard detergents for transporter structural study.

The new agents were further evaluated with a GPCR, the human β_2-adrenergic receptor (β_2AR).[64] For this experiment, DDM-purified receptor was first obtained by protein extraction from the membrane, followed by purification in 0.1% DDM. Detergent exchange from DDM into the individual GSAs (GCAs, GCEs and GDNs) was carried out by dilution. The residual DDM concentration in the sample solution was 0.0008 wt%. At a final detergent concentration of 0.2 wt%, protein stability was assessed by measuring the ability of the receptor to bind the radio-active antagonist [[^{3}H]-dihydroalprenolol (DHA)].[65] In a preliminary study where the DHA binding of the receptor was measured at 30 min after detergent exchange, all new agents except GCA-C3 were comparable to or better than DDM (Figure S8). On the basis of this preliminary result, several new

GSAs (GCA-C1/C2, GCE-C1/C3 and GDN-C1/C2/C3) were selected for further evaluation, along with a positive control of DDM. The antagonist binding of the receptor was monitored at regular intervals during a 5-day incubation at room temperature. The DDM-solubilized receptor showed a high ligand-binding ability initially, but this activity dropped rapidly with time (Figure 3). By contrast, all the selected agents were better than DDM at maintaining the ability of the receptor to bind [^{3}H]-DHA long term. The trend of detergent efficacy for receptor stabilization was as follows; the GCAs were most superior, followed by the GDNs and GCEs. The best performance was observed with GCA-C2, that yielded >80% retention of receptor activity after the 5-day incubation. This result implies that the GCAs are better alternatives to GDN and its derivatives for structural study of GPCRs.

All new agents except GCA-C3 and GCE-C2 were further evaluated with another GPCR, the mouse μ-opioid receptor (MOR).[66] Receptor stability was assessed by estimating the melting temperature (T_m) using differential scanning fluorimetry analysis. The receptor T_m in each condition was determined by finding the minimum point of the negative first-order derivative of the relative fluorescence units (-*d*RFU/*d*T) (Figure 4). The fluorescence intensity of the receptor, and thus its folded state, in the individual detergents was monitored in the presence of CPM (*N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]-maleimide) dye with increasing temperature from 15 to 70 °C (Figure S9). The individual GSAs were tested at 0.5 wt% in the presence of residual DDM. The DDM-solubilized MOR gave a low T_m (28.0 °C), indicating low stability in DDM micelles (Figure 4). When we used LMNG, a significantly improved detergent for GPCR stability, the receptor in this NG class detergent gave a T_m almost 6 °C higher (33.7 °C). Use of GDN further increased the receptor T_m to 47.7 °C, indicating that GDN has particularly stabilising effects on MOR. The new agents yielded well-defined T_m values for the receptor as indicated by the consistent CPM profiles from one experiment to another. However, CPM data for the GCA-C1-solubilzed MOR varied substantially for each experiment and thus we could not reliably calculate a T_m for this detergent-solubilized receptor (Figure S9). MOR in GDN-C2 gave a receptor T_m of 43.0 °C, indicating that this is slightly inferior to GDN. In contrast, MOR in GCA-C2 and GDN-C3 yielded higher T_m values (50.7 and 50.3 °C, respectively) than the receptor in GDN. This result clearly demonstrates the enhanced effectiveness of these steroidal detergents (GCA-C2 and GDN-C3) at stabilizing MOR compared to the gold standard single alkyl chained detergent, DDM, and the highly successful novel detergents, LMNG and GDN. Notably, all new detergents tested here gave significantly increased receptor T_m values compared to LMNG. Taken together with the results for β_2AR these findings indicate that GDN-C3 and GCA-C2 are viable and superior alternatives to the original GDN for the structural study of GPCRs.

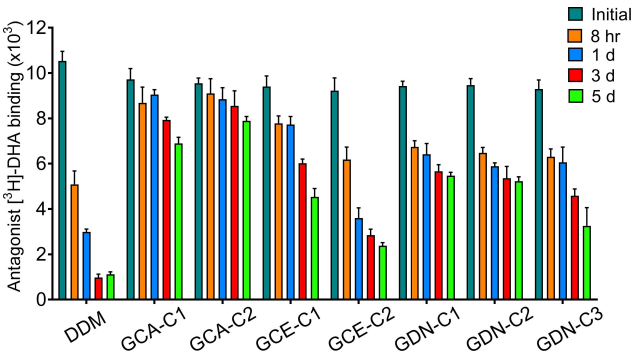


Figure 3. Long-term ligand binding ability of β_2AR solubilized in DDM and selected GSAs. DDM was used as a control agent. The detergents were tested at CMC + 0.2 wt% as detergent concentration. β_2AR stability was assessed by measuring the receptor ability to bind the radio-labelled antagonist ([^{3}H]-dihydroalprenolol (DHA)) during a 5-day incubation at room temperature. Error bars: SEM, $n = 3$.

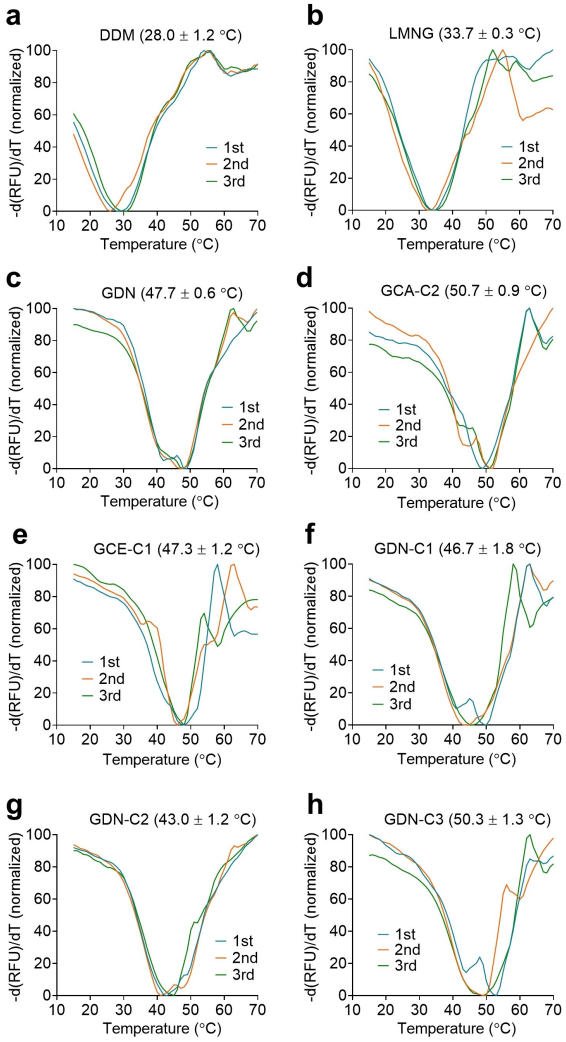


Figure 4. Normalized derivative functions of denaturation profiles obtained from a CPM-based unfolding assay of MOR solubilized in DDM (a), LMNG (b), GDN (c), GCA-C2 (d), GCE-C1 (e), GDN-C1 (f), GDN-C2 (g), or GDN-C3 (h). MOR solubilized in the individual detergents was thermally treated in the presence of CPM dye. Receptor fluorescence intensity was monitored with increasing temperature from 15 to 70 °C. The value in parentheses represents average receptor $T_m \pm$ SEM ($n = 3$–5).

The current study introduces and characterises glyco-steroidal detergents (GSAs), categorized into three sets depending on the steroidal hydrophobic group: GCAs (cholestanol), GCEs (cholesterol) and GDNs (diosgenin). The new GSAs are more accessible comparable to the original GDN in terms of the number of synthetic steps (five *vs* seven) and overall yield (~65 *vs* ~35%). Detergent synthetic accessibility is essential for widespread use of any agent in membrane protein research. These detergents were evaluated with four model membrane proteins (two transporters (MelB and LeuT) and two GPCRs (β_2AR and MOR)) and compared with DDM and the original GDN in terms of their efficacy for protein stabilization. All GSAs introduced here were more effective than DDM at stabilizing the membrane protein tested here, with the exception of GCA-C3 and GCE-C3 which were inferior to DDM at stabilizing LeuT. When compared to the original GDN, some GSAs were better at stabilizing the test proteins. For example, both GCA-C3 and GCE-C1 were more effective than GDN at retaining MelB stability at 65 °C. GCA-C2 and GDN-C3 conferred greater stability on MOR as evidenced by the increased receptor T_m values of 3.0 and 2.6 °C, respectively. The best detergents varied depending on the model membrane protein. GDN and its derivatives (GDN-C1/C2/C3) were best for LeuT stability, while the GCAs were generally better than the GDNs and GCEs for β_2AR and MOR stability, with GCA-C2 being the best agent. In the case of $MelB_{St}$, there was no clear difference in overall detergent efficacy between the GCAs, GCEs and GDNs. It is important to note that all the GDN members including the original GDN were markedly more effective at stabilizing all the membrane proteins tested here than DDM. These new detergents (GDN-C1/C2/C3 and GCA-C2) are likely to be less expensive but more effective detergents than GDN for membrane protein structural study.

Conclusions

Three sets of glyco-steroidal detergents (GSAs: GCAs, GCEs and GDNs) are more synthetically accessible than the original GDN molecule. Although inefficient at extracting MelB from the membranes, the new detergents were markedly more effective than DDM or comparable to the original GDN at stabilizing the model membrane proteins. Collectively our findings indicate that these agents have potential as biochemical tools for membrane protein structural study.

Experimental Section

Experimental details can be found in the Supporting Information, including the synthesis and characterization of the new detergents, and membrane protein stability assay.

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Data Availability Statement

The data that support the findings of this study are available in the supplementary material of this article.

Keywords: detergent design · detergent-detergent interactions · glyco-steroids · glycolipids · protein stabilization

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