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Development of 1,3-acetonedicarboxylate-derived glucoside amphiphiles (ACAs) for membrane protein study†

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Detergents are extensively used for membrane protein manipulation. Membrane proteins solubilized in conventional detergents are prone to denaturation and aggregation, rendering downstream characterization of these bio-macromolecules difficult. Although many amphiphiles have been developed to overcome the limited efficacy of conventional detergents for protein stabilization, only a handful of novel detergents have so far proved useful for membrane protein structural studies. Here, we introduce 1,3-acetonedicarboxylate-derived amphiphiles (ACAs) containing three glucose units and two alkyl chains as head and tail groups, respectively. The ACAs incorporate two different patterns of alkyl chain attachment to the core detergent unit, generating two sets of amphiphiles: ACA-As (asymmetrically alkylated) and ACA-Ss (symmetrically alkylated). The difference in the attachment pattern of the detergent alkyl chains resulted in minor variation in detergent properties such as micelle size, critical micelle concentration, and detergent behaviors toward membrane protein extraction and stabilization. In contrast, the impact of the detergent alkyl chain length on protein stability was marked. The two C11 variants (**ACA-AC11** and **ACA-SC11**) were most effective at stabilizing the tested membrane proteins. The current study not only introduces new glucosides as tools for membrane protein study, but also provides detergent structure–property relationships important for future design of novel amphiphiles.

Introduction

Membrane proteins play crucial roles in a variety of cellular functions such as cell–cell recognition, signal transduction, material transport, and cell movement. The disorder in these proteins results in various diseases such as cancers, Alzheimer's and Parkinson's.[1] In addition, these membrane-embedded bio-macromolecules are targets of more than 50% of current pharmaceuticals.[2,3] Thus, membrane protein structures are an essential part of the rational drug design and development process.[4,5] Membrane protein structures are determined through one or a combination of high-resolution techniques; X-ray crystallography, single particle cryo-electron microscopy (cryo-EM) and nuclear magnetic resonance (NMR) spectroscopy. Despite the importance of understanding structure and function of membrane proteins, the number of these bio-macromolecules with experimentally determined structure is significantly lower than that of soluble proteins.[6] This is due mainly to the inherent amphiphilic architecture of membrane proteins, making them much more likely to aggregate or denature during purification and structural studies.[7,8] Membrane-mimetic environments are necessary to preserve membrane protein structures in a state suitable for downstream characterization.[9,10] Micellar systems formed by amphiphilic molecules are an effective surrogate for the cell membrane that shields the large hydrophobic portions of membrane proteins from the aqueous environment.[11]

Small amphiphilic molecules, called detergents or surfactants, are widely used to maintain solubility and stability of membrane proteins outside their native membrane

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environment.[11–13] Detergents both disrupt the hydrophobic interactions maintaining the membrane structure and form membrane protein–detergent complexes (PDCs). Conventional detergents such as n-dodecyl-β-D-maltoside (**DDM**), n-octyl-β-D-glucoside (**OG**) and lauryldimethylamine-N-oxide (LDAO) are widely used for membrane protein extraction. Since the first structure determination of the photosynthetic reaction centre of *Rhodopseudomonas viridis* using LDAO in 1985,[14] detergents have been widely used for membrane protein structure determination. However, the canonical structure of these conventional detergents, a single hydrophobic chain and hydrophilic head group, provides insufficient structural diversity to stabilize many membrane proteins with varied structures.[10,15,16]

Over the past two decades, various approaches have been reported for the constitution of new amphipathic systems as exemplified by bicelles, membrane scaffold protein (MSP)- or styrene–maleic acid copolymer (SMA)-supported nanodiscs (NDs),[17,18] amphiphilic polymers (*e.g.*, amphipols (APols)[19]) and peptide-based detergents (*e.g.*, lipopeptide detergents (LPDs) and β-peptides (BPs)).[20,21] Although these systems are highly effective at preserving the native conformations of membrane proteins, most are ineffective at membrane protein extraction. In addition, they are often incompatible with protein crystallization.[17] Thus a great deal of effort has been devoted to developing novel detergents with enhanced efficacy for membrane protein solubilization and stabilization. A number of novel detergents including neopentyl glycol-based amphiphiles [*e.g.*, glucose neopentyl glycols (GNGs),[22–24] maltose neopentyl glycols (MNGs)[25–27] and neopentyl glycol-derived triglucosides (NDTs)[28]], rigid hydrophobic group-bearing detergents [*e.g.*, chobimalt,[29] glyco-diosgenin (GDN)[30]], facial amphiphiles (FAs)[31,32] and hemi-fluorinated surfactants (HFSs)[33] have been developed. Recently, novel detergents with distinctive hydrophilic structures were reported as exemplified by the pentasaccharide-bearing amphiphiles (PSEs)[34] and oligoglycerol detergents (OGDs).[35] In addition, stereoisomeric detergents [*e.g.*, butane-1,2,3,4-tetraol-based maltosides (BTMs),[36] norbornane-based maltosides (NBMs),[37] and cyclopentane-based maltoside (CPMs)[38]] and carbohydrate-cored amphiphiles [mannitol-based amphiphiles (MNAs),[39] scyllo-inositol-based glycosides (SIGs)[40] and trehalose-cored maltosides (TCMs)][41] have been developed to facilitate membrane protein structure study. Of these novel small amphiphiles, it is notable that MNG-3 (commercial name: lauryl maltoside neopentyl glycol (**LMNG**)) has contributed to the determination of more than 200 membrane protein structures over the last 10 years.[42] A glucoside version of **LMNG**, **OGNG** has been used for structural analysis of 17 membrane proteins.[42] In addition, new detergents play an important role in maintaining protein integrity during reconstitution of purified membrane proteins into liposomes for structural and functional analysis. The resulting proteoliposomes enable structural determination of membrane proteins in the membrane environment. In this context, it is noteworthy that the use of **LMNG** in combination with auto-insertion allowed efficient liposome reconstitution of mammalian F-ATP synthase without the loss of key subunits.[43,44] The successful examples of **LMNG** and **OGNG**

highlight the crucial role of novel amphiphiles in membrane protein structural and functional studies.

As new variants of **OGNG**, here we designed and prepared a class of detergents from a commercially available starting material, 1,3-acetonedicarboxylate. Three glucose units and two alkyl chains were attached to the 1,3-acetonedicarboxylate-derived core, resulting in detergents designated 1,3-acetonedicarboxylate-derived amphiphiles (ACAs) (Fig. 1). The alkyl chains were either attached to the central core symmetrically (ACA-Ss) or asymmetrically (ACA-As). These detergents were evaluated for membrane protein solubilization and stabilization, and a detergent comparison was made with a gold standard detergent (**DDM**) and/or two NG detergents (**OGNG** and **LMNG**). When multiple model membrane proteins were tested with these detergents, some ACAs, particularly C11 alkyl-chained detergents, conferred notably enhanced stability to the membrane proteins compared to the control agents.

Results and discussion

Detergent structures and physical characterizations

The new detergents contain two alkyl chains and three glucose units as tail and head groups, respectively. These detergents can be categorized into two sets according to the pattern of alkyl chain attachment to the central core (*i.e.*, 1,3,5-pentane-triol). For one set, the alkyl chains are attached to two different carbons of the core (2C and 4C) and for the second set, both alkyl chains are attached to the same carbon of the core. We designated these detergents as asymmetrical ACAs (ACA-As) and symmetrical ACAs (ACA-Ss), respectively (Fig. 1c and d). The ACA-As include the **OGNG** scaffold within the molecular structure, but additionally bear an ethyl glucoside appendage in the
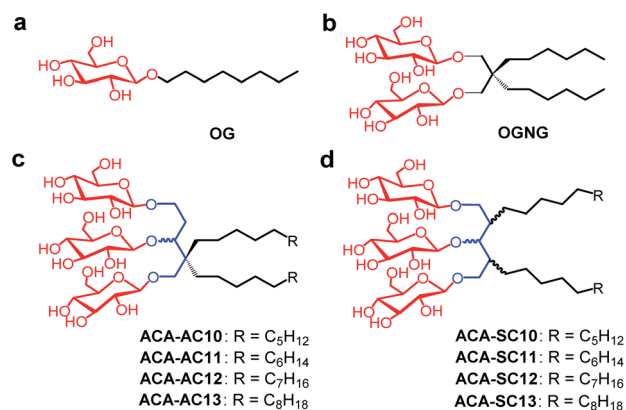

Fig. 1 Chemical structures of (a) **OG**, (b) **OGNG**, (c) ACA-As (**ACA-AC10/AC11/AC12/AC13**) and (d) ACA-Ss (**ACA-SC10/SC11/SC12/SC13**). **OGNG** has a structure that is a dimer of **OG**. The ACA-As have similar chemical structures to **OGNG**, but bear an ethyl glucoside appendage in the hydrophilic region. The ACA-Ss are structural isomers of the ACA-As. Both sets of the ACAs contain a core unit of 1,3,5-pentanetriol derived from 1,3-acetonedicarboxylate, as indicated in blue. The two alkyl chains of the ACA-As are attached at the same core carbon, whereas the different core carbons were used to attach the two alkyl chains for the ACA-Ss. The alkyl chain length of both sets varied from C10 to C13.

hydrophilic region (Fig. 1). The ACA-Ss are structural isomers of the ACA-As. The new agents contain a hydrophobic linker (*i.e.*, 1,3,5-pentane-triol) in the detergent core region, used to connect the head and tail groups. Thus, this class differs from many previous glucoside amphiphiles containing a hydrophilic linker in the same place, as exemplified by the NDTs and MNAs containing pentaerythritol and mannitol groups as linkers, respectively.[28,38] The presence of the hydrophobic linker can be favorable for membrane protein structural studies as observed with **OGNG** and **LMNG** which contain a hydrophobic NG linker. The presence of an additional glucose unit in the hydrophilic region allowed us to increase the alkyl chain length of the ACAs up to C13 with no effect on water-solubility. This is in contrast to the C6 alkyl chain of **OGNG** and the limited water-solubility (~1 wt%) of C7 alkyl-chained GNGs. The increase in the alkyl chain length compared to **OGNG** is important for optimising compatibility of detergent hydrophobic length with the hydrophobic dimensions of membrane proteins. In addition, the alkyl chain length of the ACAs varied from C10 to C13, necessary to find a detergent with an optimal hydrophilic–lipophilic balance (HLB), an important factor for stabilizing membrane proteins.[45–47]

Diethyl (or methyl) 1,3-acetonedicarboxylate was used as the starting material for preparation of the ACAs (Fig. 2a). The ACA-Ss were synthesized in four synthetic steps: alkyl chain attachment to diethyl 1,3-acetonedicarboxylate, reduction of the carbonyl functional groups to alcohol, AgOTf-promoted β-

selective glycosylation and global benzoyl group deprotection (Scheme S1†). In contrast, the ACA-As were prepared differently from the ACA-Ss. In order to attach two alkyl chains onto the same carbon of the central core, we utilized a previously reported protocol.[48,49] This asymmetric introduction of the alkyl chains into the detergent scaffold requires two additional steps for preparation of the ACA-As compared to the symmetric molecules (Scheme S2†): conversion of the central ketone group to methyl enol ether and restoration of the ketone functional group from the methyl enol ether. All the synthesized detergents were characterized by NMR spectroscopy and mass spectrometry. In the NMR spectra of the ACAs, multiple doublet signals appear in the range of 4.20 to 4.70 ppm, a peak characteristic of β-anomeric protons (Fig. 2b, c and S1†). The β-stereochemistry of the anomeric carbon can be further identified through the vicinal coupling constant (^3J). The coupling constant for the anomeric protons with the neighboring proton was observed to be 8.0 Hz, different from the typical value (4.0 Hz) for α-anomeric protons.[50] Peak complexity observed for the anomeric protons in the NMR spectra of the ACA-As and ACA-Ss is due to the presence of stereo-chemically undefined C–O and/or C–C bonds, as indicated by the wavy lines in the chemical structures (Fig. 2).

High water-solubility of a detergent is essential for their use as a biochemical tool in membrane protein applications. Both sets of detergents (ACA-Ss and ACA-As) were highly soluble in water except **ACA-SC13** and **ACA-AC13**, which gave ~5.0 and
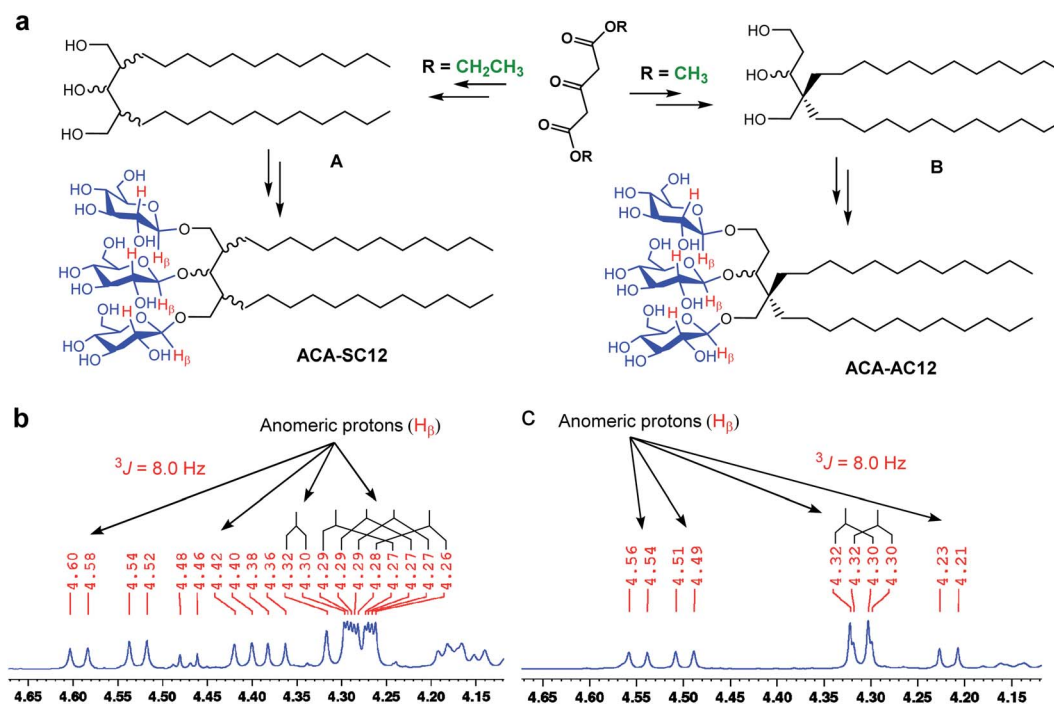

Fig. 2 (a) Schematic representation of the preparation of **ACA-SC12** and **ACA-AC12** and (b and c) their ^{1}H NMR spectra focusing on an anomeric region (4.20 to 4.70 ppm). The new detergents (**ACA-SC12** and **ACA-AC12**) were prepared from diethyl and dimethyl 1,3-acetonedicarboxylate as starting materials, respectively. The di-alkylated triol derivatives (**A** and **B**) were used to attach the three glucoside units *via* glycosylation and subsequent global deprotection. The β-stereochemistry of glycosidic bonds of the ACAs was confirmed by the NMR spectra. The peaks corresponding to the β-anomeric protons (H$_β$; red) appears in the anomeric region (4.20–4.70 ppm) having a coupling constant (^3J) of 8.0 Hz. These anomeric peaks appear as doublets, as indicated by the black bridges and arrows.

Table 1 Molecular weights (MWs), critical micelle concentrations (CMCs) of ACAs and hydrodynamic radii (R_h) (mean ± S.D., $n = 4$) of their micelles

Detergent	MW[a]	CMC (mM)	CMC (wt%)	R_h[b] (nm)	Solubility[c] (wt%)
ACA-SC10	887.1	~0.015	~0.0013	2.4 ± 0.2	>10
ACA-SC11	915.2	~0.010	~0.0009	2.9 ± 0.3	~10
ACA-SC12	943.2	~0.007	~0.0007	8.3 ± 0.1	~10
ACA-SC13	971.3	~0.005	~0.0005	13.9 ± 1.1	~5
ACA-AC10	887.1	~0.019	~0.0017	2.7 ± 0.2	>10
ACA-AC11	915.2	~0.014	~0.0013	3.3 ± 0.2	~10
ACA-AC12	943.2	~0.012	~0.0011	8.9 ± 0.7	~9
OGNG[d]	568.7	~1.0	~0.058	4.4 ± 0.3	>10
DDM	510.6	0.17	0.0087	3.4 ± 0.3	>10

[a] Molecular weight of detergents. [b] Hydrodynamic radius of micelles determined at 1.0 wt% by dynamic light scattering. [c] Water-solubility at room temperature. [d] Data obtained from a literature.[22]

<1.0 wt% water-solubility, respectively (Table 1). Due to this low water-solubility, **ACA-AC13** was not further tested. Self-assembly properties of the individual detergents were investigated by measuring critical micelle concentrations (CMCs) and hydrodynamic radii (R_h) of their micelles formed in aqueous solution.[51,52] All ACAs exhibit lower CMCs than **DDM** and **OGNG** (0.005–0.019 mM *vs.* 0.17 mM (**DDM**) and 1.0 mM (**OGNG**)), indicating strong hydrophobic interaction between the ACA detergent molecules. The increased hydrophobic interaction is mainly due to either the presence of two alkyl chains or of elongated alkyl chains. Detergent micelles of the ACAs varied from 2.4 nm to 13.9 nm depending on detergent symmetricity and alkyl chain length (Table 1). The micelle sizes of **ACA-SC10/11**, and **ACA-AC10/11** were smaller than those of **DDM** and **OGNG**, while the micelles formed by the C12/C13 alkyl-chained ACAs were significantly larger compared to **DDM**, **OGNG** and the C10/C11 alkyl chain versions. Notably, the ACA-Ss gave lower CMCs and smaller micelle sizes than the ACA-As. For example, **ACA-SC11** gave a lower CMC than **ACA-AC11** (0.010 *vs.* 0.014 mM) and the micelle size of **ACA-SC11** was smaller than that of **ACA-AC11** (2.9 *vs.* 3.3 nm). These features are likely to be associated with the enhanced water-solubility of the ACA-Ss compared to their asymmetric counterparts; **ACA-SC13** gave a higher water-solubility than **ACA-AC13** (~5 *vs.* <1.0 wt%). Detergent micelle size tends to increase with increasing alkyl chain length. A dramatic increase in detergent micelle size was observed, particularly when the alkyl chain length increases from C11 to C12 in both series (Table 1 and Fig. S2†). This correlation between detergent alkyl chain length and micelle size can be explained by a change in detergent molecular shape accompanied by increasing alkyl chain length. The incorporation of an additional carbon into the hydrophobic tail results in a detergent molecule with a more cylindrical shape, favorable for formation of large self-assemblies.[53] Dynamic light scattering profiles indicate little polydispersity for self-assemblies formed by the ACA-Ss/As (Fig. S2†).

Detergent evaluation for membrane protein stability

The ACAs were first evaluated with LeuT from the bacterium, *Aquifex aeolicus*.[54] The transporter was first extracted from *E. coli* using 1.0 wt% **DDM**, followed by purification with 0.05 wt% of the same detergent. The **DDM**-purified LeuT was diluted into buffer solution containing **DDM** or the respective ACA-Ss/As to give a final detergent concentration of CMC + 0.04/0.2 wt% (Fig. 3). Protein stability is assessed by measuring the ability of the transporter to bind a radio-labeled substrate ([³H]-Leu) *via* scintillation proximity assay (SPA).[55] The substrate binding ability was monitored at regular intervals over a 13 day incubation period at room temperature. At the detergent concentration of CMC + 0.04 wt%, initial activity (*i.e.*, ligand binding) of LeuT solubilized in **DDM** was high compared to those of the ACAs, but this initial activity of the transporter in this detergent gradually decreased over time (Fig. 3a). In contrast, the ACA-solubilized transporters tended to give rather low initial [³H]-Leu binding compared to the **DDM**-solubilized protein, but binding activity recovered within two days (Fig. 3). Four ACA agents (**ACA-AC10**, **ACA-AC11**, **ACA-AC12**, and **ACA-SC12**) were clearly better than **DDM** at maintaining long-term LeuT stability under the conditions tested (Fig. S3 and Table S1†). At an increased detergent concentration of CMC + 0.2 wt%, the ACA efficacies for LeuT stabilization were in most cases enhanced compared to those obtained at CMC + 0.04 wt% (Fig. 3b). This enhancement was particularly notable for **ACA-SC13**. As
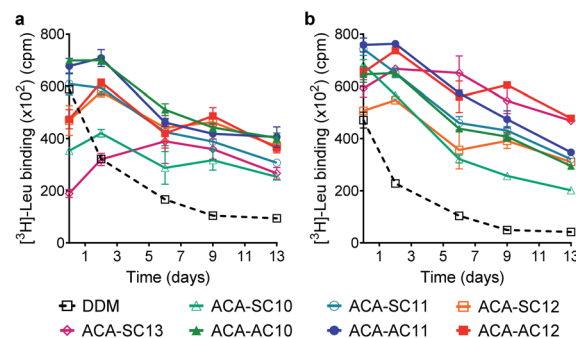

Fig. 3 Long-term stability of LeuT solubilized in the ACA-Ss and ACA-As at CMC + 0.04 wt% (a) and CMC + 0.2 wt% (b). **DDM** was used as a control agent. For LeuT stability assessment, the ability of the transporter to bind the radiolabelled substrate ([³H]-leucine) was monitored at regular intervals during a 13 day incubation at room temperature *via* scintillation proximity assay (SPA). Data points are mean ± SEM (error bars); $n = 3$–6.

a results, all ACAs were superior to **DDM** for LeuT stability under the conditions tested (Fig. S3 and Table S1†). Taken together these results reveal that all the ACA-solubilized transporters were more effective than **DDM**-solubilized protein at retaining Leu binding over the 13 day incubation period. The best performance was observed with **ACA-AC11/AC12**, followed by **ACA-SC11** and **ACA-SC13**. There is no notable difference in detergent efficacy between the two series (ACA-As and ACA-Ss). Of note, **OGNG** was previously reported to be inferior to **DDM** for LeuT stability.[22,56] Thus, these results indicate the potential utility of the ACAs in structural studies of membrane transporters.

For further evaluation of the new detergents, we used the melibiose permease from *Salmonella typhimurium* (MelB$_{St}$).[57–60] MelB$_{St}$ expressed in *E. coli* membranes was extracted using 1.5 wt% of each detergent (**DDM**/ACAs) for 90 min at 0 °C. The protein extracts were further incubated at an elevated temperature (45, 55, or 65 °C) for another 90 min. The amounts of soluble MelB$_{St}$ obtained in each individual condition were assessed by SDS-PAGE and Western blotting and presented as percentages (%) of the membrane-bound transporter (Fig. 4a). MelB$_{St}$ extraction at 0 °C was carried out to estimate detergent efficiency for protein extraction. Further incubation of the MelB$_{St}$ extracts at elevated temperatures provides further information on detergent efficacy for protein stabilization. At 0 °C, **DDM** was efficient at protein extraction, yielding nearly 100% soluble MelB$_{St}$. The long-alkyl chained ACAs such as **ACA-AC12**, **ACA-SC12**, and **ACA-SC13** yielded much lower amounts of soluble MelB$_{St}$ (less than 40%). In contrast, the C10 and C11 alkyl-chained ACAs were comparable to **DDM** (Fig. 4a and Table S2†). A similar trend was observed when the samples were further treated at 45 °C (Table S3†). The only difference was a slight increase in the amount of soluble MelB$_{St}$ in the case of **ACA-SC12/AC12**. When the temperature was further increased to 55 °C, a dramatic difference in the amount of soluble MelB$_{St}$ was found between the tested detergents (Table S4†). **ACA-AC10** and **ACA-SC13** yielded ∼10% soluble MelB$_{St}$, similar to **DDM**, whereas **ACA-AC11** and **ACA-SC11** gave ∼70% and ∼60% soluble MelB$_{St}$, respectively. Of note, it was reported that **OGNG** is inferior to **DDM** for MelB stability in previous studies.[22,56] It is interesting to note that the ACAs behaved differently depending on the alkyl chain length. Specifically, the C10 alkyl chain members were as efficient as the C11 versions at protein extraction, but were less effective than the latter in terms of MelB stability. An opposite trend was observed for the C12 alkyl-chained ACAs. These detergents were inefficient at MelB extraction, but effective at maintaining MelB stability. The C11 alkyl-chained ACAs (**ACA-AC11** and **ACA-SC11**) were not only efficient at MelB$_{St}$ extraction, but were also effective at maintaining this protein in a soluble state at elevated temperature. Thus, these two detergents were selected to further evaluate their abilities to maintain MelB$_{St}$ functionality. MelB$_{St}$ functionality was assessed by monitoring fluorescence intensity changes associated with the ability of the transporter to bind the substrate (melibiose) and fluorescent ligand (dansyl-2-galactoside (D^{2}G)).[61] A high fluorescence intensity would be observed for functional transporter in the presence of D^{2}G as
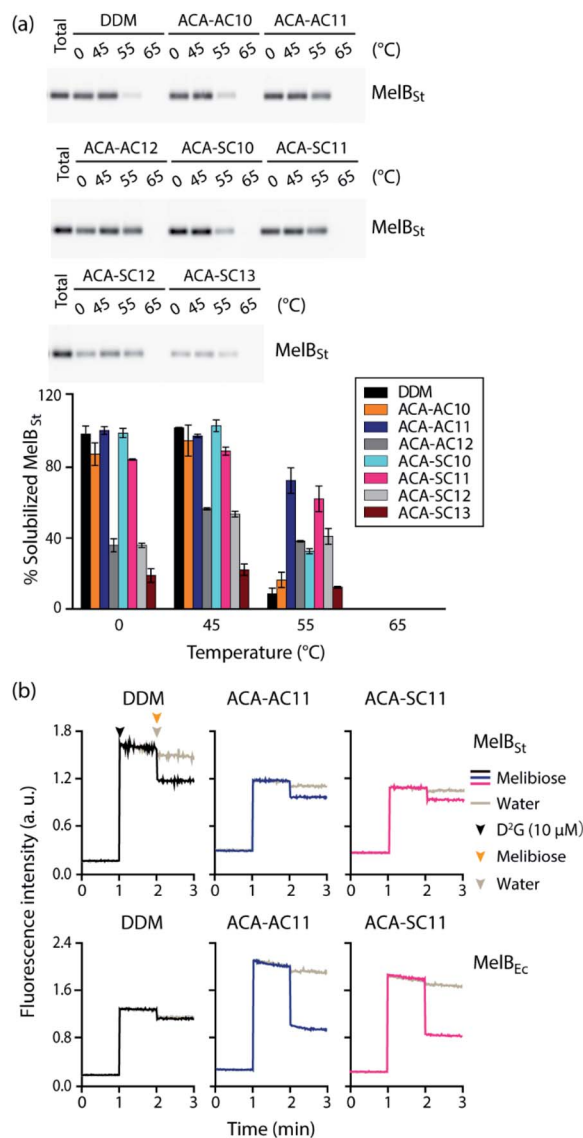

Fig. 4 Thermostability of MelB$_{St}$ solubilized in the new agents (ACA-As and ACA-Ss) (a) and MelB$_{St}$ functionality in **ACA-AC11** and **ACA-SC11** (b). **DDM** was used as a control. 1.5 wt% of each detergent was used for protein extraction at 0 °C and the resulting MelB$_{St}$ extracts were further incubated at 45, 55 or 65 °C for another 90 min. (a) After being subjected to ultracentrifugation, the individual samples were analyzed using SDS-PAGE and western blotting to estimate the amounts of soluble MelB$_{St}$. The amounts of soluble MelB$_{St}$ are represented as relative percentages (%) of total MelB$_{St}$ present in untreated membranes (expressed as 'total') in the histogram. Error bars, SEM; $n = 2$. The data were statistically analysed using one-way ANOVA (Tables S1–S3†). (b) MelB function was assessed by melibiose-mediated FRET reversal. Dansyl-2-galactoside (D^{2}G) and excess melibiose were added to **ACA-AC11/SC11**-extracted MelB (MelB$_{St}$ and MelB$_{Ec}$) at 1 min and 2 min time points, respectively. Fluorescence intensity was monitored over the course of the additions of the ligand and substrate. Control data was obtained by addition of water instead of melibiose. Melibiose addition is indicated in black (**DDM**), blue (**ACA-AC11**), and purple (**ACA-SC11**), and water addition is indicated in gray.

the result of efficient Förster resonance energy transfer (FRET) from tryptophan (Trp) to D^{2}G. Subsequent addition of melibiose results in a decrease in fluorescence intensity as this non-

fluorescent substrate replaces D^{2}G in the active site. Thus, monitoring fluorescence intensity over the course of D^{2}G and melibiose additions allowed assessment of MelB functionality solubilized in the individual detergents. The **DDM**-extracted MelB$_{St}$ retained functionality, as the increase and decrease in the fluorescence intensity were detected upon addition of first D^{2}G and then melibiose (Fig. 4b). When a less stable MelB homologue from *E. coli* (MelB$_{Ec}$) was employed, however, **DDM** failed to yield a relevant fluorescence intensity in response to addition of D^{2}G or melibiose. This result indicates that **DDM**-solubilized MelB$_{Ec}$ had lost its sugar binding function, in marked contrast to the retention of protein function observed for **ACA-AC11** and **ACA-SC11** under the same conditions. Therefore, **ACA-AC11** and **ACA-SC11** are superior to **DDM** in maintaining MelB in a soluble and functional state.

In order to evaluate the new detergents for G protein-coupled receptor (GPCR) stability, the human β$_2$ adrenergic receptor (β$_2$AR) was used for assessment.[62] **DDM**-purified receptor was exchanged into each detergent (*i.e.*, **DDM**, **OGNG**, **LMNG**, ACA-Ss/As) *via* dilution. The final detergent concentration was 0.2 wt%. During a 7 day incubation at room temperature, we monitored the specific ligand binding ability of the receptor at regular intervals using the radio-labelled antagonist ([^{3}H]-dihydroalprenolol (DHA)) (Fig. 5a).[63] The receptor solubilized in **DDM** showed low ligand binding upon detergent exchange, also observed for **OGNG**-solubilized receptor. This low level of ligand binding is likely to indicate partial denaturation of the receptor in **DDM** micelles. When exchanged into the ACAs, the initial ability of the receptor to bind DHA increased significantly compared to **DDM/OGNG**. This enhanced DHA binding is likely due to refolding of the 'partially-denatured' receptor by the favourable action of the new detergents on the receptor, rescuing the receptor ability to bind the DHA ligand. The initial receptor activity (*i.e.*, DHA binding) tended to decrease with increasing detergent alkyl chain length. Consequently, C10 and C11 alkyl-chained detergents showed initial high activity, while the C12 and C13 alkyl-chained ACAs gave intermediate activity upon detergent exchange. Interestingly, an opposite trend was obtained for detergent efficacy for long-term receptor stabilization. The C11/C12 alkyl-chained ACAs were better than the C10 alkyl-chained detergents in this regard (Fig. S4a, c and Table S5†). Combined together, **ACA-SC11** and **ACA-AC11** were the best of the ACAs and these ACAs were substantially better than or comparable to **LMNG**, a significantly optimized detergent for GPCR stability (Fig. 5a) (Fig. S4a, c and Table S5†). These detergents retained 40∼50% initial receptor activity after the 7 day incubation, while **LMNG** gave only 10% retention under the same conditions (Fig. S5a†). Thus, this result indicates that these detergents could be useful for GPCR structural studies.

Detergent evaluation was continued with another GPCR, the mouse μ-opioid receptor (MOR).[64] The receptor was first purified in **LMNG** and exchanged into the respective ACA *via* dilution. The final detergent concentration was 0.1 wt%. MOR stability was measured by assessment of radioactive antagonist ([^{3}H]-diprenorphine (DPN)) binding at regular intervals during a 5 day incubation at 4 °C (Fig. 5b).[65] The receptor solubilized in
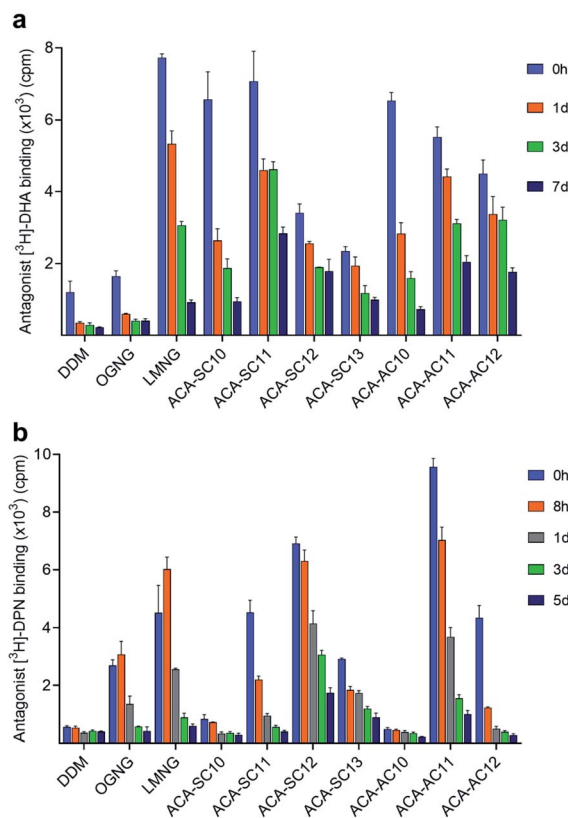

Fig. 5 Evaluation of the new detergent for (a) β$_2$AR and (b) MOR stability. DDM, OGNG and LMNG were used as control agents. β$_2$AR and MOR purified in DDM and LMNG, respectively, were diluted into a buffer containing the indicated detergents to reach final detergent concentrations of 0.2 and 0.1 wt%. The receptor ability to specifically bind a radioactive antagonist ([^{3}H]-dihydroalprenolol (DHA) (β$_2$AR) or [^{3}H]-diprenorphine (DPN) (MOR)) was monitored at regular intervals during a 7 day incubation at room temperature (β$_2$AR) or a 5 day incubation at 4 °C (MOR). Data points are mean ± SEM (error bars); $n = 3$.

DDM appeared to lose the ability to specifically bind ligand completely upon detergent exchange. Similar results were observed for the two C10 alkyl-chained ACAs, **ACA-SC10** and **ACA-AC10** (Fig. 5b). Interestingly, **OGNG** yielded the receptor with enhanced DPN binding compared to **DDM** (Fig. S4b, d and Table S6†). Use of **LMNG** led to a further increase in the initial receptor activity, but, similar to **ACA-SC11** and **ACA-AC12**, the receptor solubilized in this maltoside exhibited rapid loss in DPN binding. The receptor in **LMNG** gave only 10% retention in the ligand binding of the receptor after the 5 day incubation (Fig. 5b). Remarkably, the receptors encapsulated in **ACA-SC12/AC11** micelles not only showed higher initial activity than the **LMNG**-solubilized protein, but were more effective than the latter at preserving DPN binding capability long term (Fig. S4b, d and Table S6†). **ACA-SC12**-solubilized receptor retained ∼30% of initial ligand binding after the 5 day incubation (Fig. S5b†). Combined with the β$_2$AR result, these findings for the MOR indicate that **ACA-AC11**, **ACA-SC11**, and **ACA-SC12** hold significant potential for GPCR structural study.

In order to further explore whether the new detergents can be used for membrane protein extraction, we selected four ACAs

(**ACA-AC10**, **ACA-SC10**, **ACA-AC11**, and **ACA-SC11**) for extraction of two GPCRs (β₂AR and MOR). These detergents were selected as they generated large amounts of soluble MelB$_{St}$, close to levels obtained with **DDM** (Fig. 4). **DDM** and **LMNG** were used as controls as these detergents are widely used for GPCR extraction.[66] When tested for β₂AR extraction, **DDM** extracted the receptor quantitatively from the membranes (Fig. S6a†). Use of **LMNG** resulted in a large amount of soluble β₂AR (∼80%), although slightly less than **DDM**. Of the tested ACAs, **ACA-SC11** yielded the largest amount of soluble β₂AR, followed by **ACA-AC10** and **ACA-SC10**. The amount of β₂AR solubilized by **ACA-SC11** was more or less comparable to that achieved in **DDM/LMNG**. In contrast, the asymmetric version, **ACA-AC11**, yielded only low levels of soluble β₂AR. A different trend was observed for MOR extraction. **LMNG** was slightly more effective than **DDM** at extracting this GPCR, likely related to the fact that MOR is more stable in **LMNG**. All tested ACAs except **ACA-SC11** yielded similar levels of soluble receptor to **DDM**. Notably, **ACA-AC11**-solubilized MOR showed little protein aggregation, while **DDM/LMNG**-solubilized receptor appeared to undergo substantial aggregation (Fig. S6b†). Combined with the MelB$_{St}$ results, these findings demonstrate that the new detergents can be used for membrane protein extraction as efficiently as **DDM/LMNG**.

Membrane proteins have diverse structures and thus their hydrophobic and hydrophilic dimensions vary. Consequently, these bio-macromolecules vary in terms of their tendency to denature and aggregate. The varied dimensions and diverse properties of membrane proteins imply a protein-specific nature of detergent efficacy for protein stabilization, as observed in the current study. For instance, **ACA-AC12** was the most effective ACA at stabilizing LeuT, but this C12 alkyl-chained detergent was poor at stabilizing MOR. As another example, **ACA-SC11** was inferior to **ACA-SC12** at stabilizing MOR, but was the most effective of the ACA-Ss at stabilizing the other tested membrane proteins (LeuT, MelB and β₂AR). Despite this apparent protein-specific nature of the detergents, **ACA-AC11** was superior to **DDM** for stabilization of all the tested membrane proteins. The symmetric version of this detergent, **ACA-SC11**, also exhibited generally favourable behaviour toward stabilization of all tested membrane proteins except MOR. Notably, these C11 alkyl-chained ACAs (**ACA-AC11** and **ACA-SC11**) showed enhanced GPCR stability even when compared to **LMNG**. This is a remarkable feature as glucoside detergents such as **OG** and **OGNG** are generally inferior to their maltoside counterparts (**DDM** and **LMNG**) with respect to protein stability. Furthermore, these C11 alkyl-chained ACAs (**ACA-AC11** and **ACA-SC11**) gave high protein extraction efficiencies; **ACA-AC11** and **ACA-SC11** gave highly efficient MelB$_{St}$ extraction, comparable to **DDM**, and were as efficient as **DDM** and **LMNG** at extracting β₂AR and MOR, respectively. Thus, these detergents have potential in protein extraction, purification and structural studies.

Detergent efficacy for membrane protein stabilization tends to be significantly affected by a minor variation in detergent structure. In the current study, however, we could not find a meaningful difference in detergent efficacy for protein stabilization between the ACA-Ss and ACA-As. For instance, the ACA-As were generally better than their symmetric counterparts for LeuT

stability, but the relative efficacy of these two sets for MOR stabilization varied depending on detergent alkyl chain length. For example, **ACA-AC11** was better than **ACA-SC11** at stabilizing this GPCR, whereas the opposite trend was observed for the C12 alkyl-chained ACAs. In the case of stability of β₂AR and MelB, there was no substantial difference in detergent efficacy between the two sets of ACAs. This is somewhat unexpected considering their obvious structural differences. As the two alkyl chains were attached either to the same carbon or to different carbons of the central core, their alkyl chain density should be different. The alkyl chain density appears to be higher for the ACA-Ss, as supported by the lower CMCs and smaller micelle sizes compared to the ACA-As. In addition, due to the presence of the ethyl glucoside appendage, the hydrophilic group of the ACA-As is likely to be more flexible than that of the symmetric versions. The minor efficacy difference between the symmetric and asymmetric versions, despite the presence of their obvious structural differences, is likely attributed to the fact that several factors such as HLB, alkyl chain density/inter alkyl chain distance, and flexibility of detergent head group collectively affect detergent behaviour for protein stability. Thus, it is possible that one advantageous feature (*e.g.*, flexibility of the hydrophilic group) is offset by detrimental feature (*e.g.*, low alkyl chain density) for protein stability in the same set of detergents. A further study is necessary to explore this hypothesis.

It is noteworthy that detergent alkyl chain length was found to be highly important for protein stability in the current study. Of the ACA-As, the C11 version (**ACA-AC11**) was most effective at stabilizing the tested membrane proteins, followed by the C12 and C10 versions. A similar tendency was observed for the ACA-Ss. The only exception was MOR which was more stable in **ACA-SC12** than the C11 version. As membrane proteins have a narrow range of hydrophobic widths (28–32 Å), the detergent alkyl chain length that is optimal for membrane protein stability should be limited.[67,68] Furthermore, the alkyl chain length plays a central role in detergent hydrophobicity, a feature that strongly influences *detergent–detergent* and *detergent–protein* interactions in PDC environments. The short alkyl-chained ACAs (**ACA-AC10** and **ACA-SC10**) are likely form relatively weak *detergent–detergent* and *detergent–protein* interactions, and are thus unlikely to be effective at preventing protein aggregation. In addition, the C10 alkyl chain of these detergents seems to be too short to effectively cover the hydrophobic surfaces of the membrane proteins. In contrast, the C12 alkyl-chained ACAs should support strong *detergent–detergent* and *detergent–protein* interactions, and therefore effective at preventing membrane protein aggregation. When it comes to compatibility with a protein's hydrophobic dimensions, however, the C12 alkyl-chain ACAs may be suboptimal due to the formation of micelles with large hydrophobic dimensions.[69] The current study showed that the C11 alkyl-chained ACAs were best for protein stability in most cases, indicating that the C11 alkyl chain in the ACA architecture is hydrophobic enough to prevent protein aggregation and optimal for effective encapsulation of the hydrophobic surfaces of membrane proteins. It is notable that the C11 alkyl chain length has been found to be most effective for protein stability in the cases of other amphiphiles.[28,34]

Conclusions

Two sets of glucoside amphiphiles were designed and prepared with length and positional variations of the alkyl chains. These detergents (ACA-As and ACA-Ss) were evaluated with two transporters (LeuT and MelB) and two GPCRs (β_2AR and MOR) in terms of protein solubilization and stabilization. The C11 versions (**ACA-AC11** and **ACA-SC11**) are not only as efficient as **DDM** for protein extraction, but are also superior to the latter for membrane protein stabilization. Although they are glucoside detergents, **ACA-AC11** and **ACA-SC11/SC12** conferred markedly enhanced stability compared to the maltoside detergent (**LMNG**). Several advantageous features of these ACAs, such as convenient synthesis, high efficiency for membrane protein extraction and enhanced efficacy for membrane protein stabilization indicate their significant potential for the membrane protein manipulation and structural studies. The detailed analysis of the structure–efficacy relationship of the detergents provided here should facilitate new detergent design and development for membrane protein studies.

Data availability

The data that support the findings of this study are available in the ESI† of this article.

Author contributions

P. S. C. designed the new amphiphiles. H. J. L. and M. E. synthesized the amphiphiles. H. J. L., M. E., X. Z., S. K., C. F. M., H. W., W. A., and A. K. designed and performed the research and interpreted the data. P. S. C., X. L., L. G., C. J. L., and B. B. contributed to experimental design and data interpretation. P. S. C. and H. J. L. wrote the manuscript, with oversight from all the other authors.

Conflicts of interest

The authors declare the following competing financial interest(s): P. S. C., H. J. L., and M. E. are inventors on a patent application describing the ACAs.

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