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Novel Xylene-Linked Maltoside Amphiphiles (XMAs) for Membrane Protein Stabilisation

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Abstract: Membrane proteins are key functional players in biological systems. These biomacromolecules contain both hydrophilic and hydrophobic regions and thus amphipathic molecules are necessary to extract membrane proteins from their native lipid environments and stabilise them in aqueous solutions. Conventional detergents are commonly used for membrane protein manipulation, but membrane proteins surrounded by these agents often undergo denaturation and aggregation. In this study, a novel class of maltoside-bearing amphiphiles, with a xylene linker in the central region, designated xylene-linked maltoside amphiphiles (XMAs) was developed. When these novel agents were evaluated with a number of membrane proteins, it was found that XMA-4 and XMA-5 have particularly favourable efficacy with respect to membrane protein stabilisation, indicating that these agents hold significant potential for membrane protein structural study.

All cells are surrounded by plasma membranes, comprising lipid molecules and membrane proteins. Animal cells contain additional intracellular compartments including the nucleus, endoplasmic reticulum, Golgi apparatus, lysosomes and mitochondria (or chloroplasts in plant cells). The individual functions of these organelles allow synchronized cellular activity in a controlled environment. Membrane proteins inserted in the lipid bilayers of both cells and organelles are involved in numerous biological processes. For instance, they transport a variety of versatile biomolecules ranging from small ions or molecules to large proteins and nucleic acids (DNAs and RNAs). Membrane proteins also mediate the transfer of information across the membrane, allowing cells to respond to a variety of environmental stimuli. Cell-to-cell communication mediated by membrane proteins is essential to arrange activity of a number of cells constituting individual tissues and organs. Due to these essential biological roles, membrane proteins are the targets of about 50% of the currently available pharmaceuticals.[1] High resolution structural studies of membrane proteins are essential to both gain insight into the mechanisms of action of these important molecules and also to provide information for rational drug design.[2] However, membrane protein structural study lags far behind that of soluble proteins. Currently, only approximately 1% of proteins of known structure are membrane proteins,[3] highlighting that structural study of membrane proteins is extremely challenging. This is mainly due to the incompatibility of the large hydrophobic protein surface with the polar environment of an aqueous medium.[4] Amphipathic agents, called detergents, are commonly used to overcome such incompatibility, as exemplified by the popular use of *n*-octyl-β-D-glucoside (OG), lauryldimethylamine-*N*-oxide (LDAO), and *n*-dodecyl-β-D-maltoside (DDM).[5] However, most detergent-solubilized membrane proteins exhibit limited structural stability.[6] Therefore, it is of great interest and importance that novel classes of amphiphiles, with enhanced membrane protein stabilisation efficacy, are developed in order to provide additional, improved tools for membrane protein research.[7]

Conventional detergents typically comprise a single flexible alkyl chain attached to a large hydrophilic head group.[5,8] Owing to the low structural diversity of conventional detergents, they have a limited range of micellar properties, which in turn limits their ability to prevent protein aggregation and denaturation. Furthermore, despite more than 120 conventional detergents being available, only a handful of detergents are widely used for membrane protein study. Membrane proteins encapsulated even by these popular detergents tend to undergo structural degradation, hampering advances in membrane protein structural study.[9] To cope with a large diversity of membrane proteins with a range of tendencies to either

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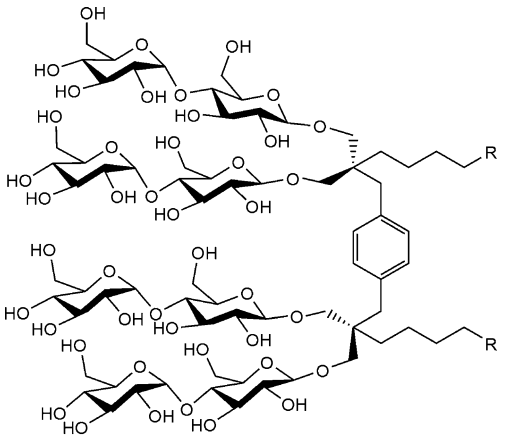
aggregate and/or denature, novel amphipathic agents with greater structural variations need to be developed.

Over the last two decades, a number of novel agents have been described which can be divided into four categories; variants of conventional detergents, peptide-based amphiphiles, membrane-mimetic systems with an amphipathic polymer, and rigid hydrophobic group-bearing agents. Chae's Glyco–Tritons (CGTs)[10a] and deoxycholate-based *N*-oxides (DCAOs),[10b] variants of Triton X-100 and 3-[(3-cholamindopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), respectively, show favourable behaviour compared to the parent compounds for membrane protein solubilisation/stabilisation. Peptide-based amphiphiles are represented by lipopeptide detergents (LPDs),[11a] short peptides,[11b] and beta-peptides (BPs).[11c] More complex systems utilizing amphiphilic polymers (e.g., amphipols (Apols),[12a,b] nanodiscs (NDs),[12c] nanolipodisqs[12d]) show promising protein stabilisation efficacy for a number of membrane protein systems. Apols and nanolipodisq particles have a flexible poly(acrylic acid) (PAA) and styrene-maleic acid (SMA) polymer backbone, respectively, while two amphipathic peptide chains derived from human high density lipoprotein apoA-1 protein were used to generate NDs. However, these peptide-based agents and polymer-based membrane-mimetic systems are yet to contribute to membrane protein structural study.

The most encouraging results have been obtained using rigid hydrophobic group-bearing agents, represented by the successful cases of facial amphiphiles (FAs)[13a,b] and the neopentyl-glycol (NG) amphiphiles (glucose neopentyl-glycols (GNGs)[13c,d] and maltose neopentyl-glycols (MNGs)[13e–g]). The FAs, GNGs and MNGs have contributed to the high resolution structure determination of various membrane proteins. MNG-3 (a.k.a., LMNG), has been particularly successful, with the facilitation of more than 20 new crystal structures of membrane proteins during the last four years.[14a–q] More representatives of rigid hydrophobic group-bearing agents include tripod amphiphiles (TPAs),[15a–c] chobimalt,[15d] glyco-diosgenin (GDN),[15e] and adamantane-based amphiphiles (ADAs).[15f] In this study, we designed and prepared a novel class of amphiphiles with a *p*-dimethylbenzene (i.e., *p*-xylene) linker, designated xylene-linked maltoside amphiphiles (XMAs; Scheme 1). These novel agents with a rigid core and flexible tails were characterized in terms of their ability to stabilise membrane proteins using four different membrane proteins. The results showed that two new agents, XMA-4 and XMA-5, are comparable or superior to DDM and the other XMAs for most of the membrane proteins tested.

The new agents bear two alkyl chains as the hydrophobic groups and four maltosides as the hydrophilic groups (Scheme 1). Two quaternary carbons located between the alkyl chain and the maltoside head groups were connected via a rigid linker, a *p*-xylene group. Thus, these XMAs have a rather rigid core structure together with flexible alkyl chains. This rigid core could facilitate crystal lattice formation through the formation of a more ordered protein–detergent complex.[15a] The new amphiphiles have variations in the alkyl chain length; XMA-1, XMA-2, XMA-3, XMA-4, and XMA-5 contain C8, C9, C10, C11 and C12 alkyl chains, respectively. These compounds were prepared in five synthetic steps; monoalkylation of diethylmalonate, coupling with *p*-bis(bromomethyl)benzene, reduction of the ester functional group, glycosylation and deprotection (see the Supporting Information for details). Because of the high efficiency of each synthetic step, the final amphipathic compounds could be prepared with overall yields of approximately 60%, making preparation of multigram quantities of the materials feasible.



XMA-1 : R = *n*-C_4H_9

XMA-2 : R = *n*-C_5H_{11}

XMA-3 : R = *n*-C_6H_{13}

XMA-4 : R = *n*-C_7H_{15}

XMA-5 : R = *n*-C_8H_{17}

Scheme 1. Chemical structures of newly prepared xylene-linked maltoside amphiphiles (XMAs; XMA-1, XMA-2, XMA-3, XMA-4, and XMA-5).

All new agents were water-soluble up to 10%. In the case of XMA-5, a brief sonication was required to generate a clear 10% aqueous solution. The critical micellar concentrations (CMCs) of the XMAs were determined by using a fluorescent dye, diphenylhexatriene (DPH),[16] and the hydrodynamic radius (R_h) of micelles formed by each agent was estimated through dynamic light scattering (DLS) experiments. The summarized results are presented in Table 1. The CMC values of the XMAs (from 1 to 20 μM) turned out be much smaller than that of DDM (170 μM) and tended to decrease with increasing detergent alkyl chain length; XMA-1 and XMA-5 with the shortest and longest alkyl chain length (C8 and C12) were estimated to give CMC values of approximately 20 μM (~0.004 wt%) and 1 μM (~0.0002 wt%), respectively. The relatively small CMC values of XMAs imply a strong tendency to self-aggregate and mean that smaller amounts of material are needed than DDM for some applications (e.g., detergent exchange). The sizes of micelles formed by XMAs tend to increase with the alkyl chain length, giving the smallest and largest micelle sizes for XMA-1 (2.7 nm) and XMA-5 (3.7 nm), respectively. In terms of micelle size, XMA-1 and XMA-2 were smaller than DDM while XMA-3 and XMA-4 were comparable to DDM. When we investigated

the size distribution for XMA micelles, XMA-1 and XMA-2 showed only one population of micelles, as does DDM, while XMA-3, XMA-4, and XMA-5 gave two populations of micelles (Figure S1 in the Supporting Information) with very different radii. The number ratios for the two sets of micelles were estimated to be more than 10^6 given the fact that the intensity of the scattered light is proportional to the sixth power of the micelle radius.[17] Thus, the set of micelles with smaller size is an almost exclusive entity present in an amphiphile solution containing XMA-3, XMA-4, or XMA-5.

Table 1. Molecular weights and critical micelle concentrations (CMCs) of XMAs and a conventional detergent (DDM), and the hydrodynamic radii (R_h; $n=5$) of their micelles.

Detergent	M_W[a]	CMC [μM]	CMC [wt%]	R_h [nm][b]
XMA-1	1775.9	~20	~0.004	2.7±0.04
XMA-2	1803.9	~10	~0.002	3.2±0.01
XMA-3	1832.0	~7	~0.001	3.5±0.01
XMA-4	1860.0	~3	~0.0006	3.3±0.03
XMA-5	1888.1	~1	~0.0002	3.7±0.01
DDM	510.1	~170	~0.0087	3.4±0.02

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

The new XMAs were first evaluated with Bor1, a boron transporter from *Arabadopsis thaliana*, expressed in *Saccharomyces cerevisiae*.[18] The protein was initially solubilised, purified and concentrated to 7 mg mL^{-1} in DDM prior to detergent exchange by a process of dilution (1:100) into solutions containing the individual XMAs. Protein unfolding (i.e., denaturation) was monitored using a fluorescent dye, *N*-[4-(7-diethylamino-4-methyl-3-coumarinyl)phenyl]maleimide (CPM)[19] and this thermal denaturation assay was carried out at 40 °C for 120 min. Upon protein denaturation, the sulfhydryl group of cysteine amino acid residues become solvent-accessible and thus react with the maleimide group of CPM dye, increasing its fluorescence intensity. As DDM was the most destabilising condition for Bor1 after 2 h incubation at 40 °C, the amounts of folded protein present in individual XMA solutions were normalized relative to that (Figure 1). In order to investigate the detergent concentration effect on protein stability, two concentrations were used for this assay; CMC + 0.04 wt% and CMC + 0.2 wt%. At both concentrations all XMAs were superior to DDM. However, it was difficult to identify the best XMA for Bor1. All the XMAs stabilised the protein to a similar extent and there was some inter-assay variability with respect to which of the new agents conferred the greatest stability.

The new agents were further characterized with *Salmonella typhimurium* melibiose permease ($MelB_{St}$), which catalyses the symport of a galactopyranoside and a coupling cation (H^+, Li^+, or Na^+).[20] In order to investigate detergent efficacy for $MelB_{St}$ solubilisation and stabilisation, *E. coli* membranes expressing $MelB_{St}$ at 10 mg mL^{-1} were treated with 1.5 wt% individual detergent solutions at the four different temperatures (0, 45, 55, and 65 °C) for 90 min. After ultracentrifugation, the amount of soluble protein from each condition was estimated

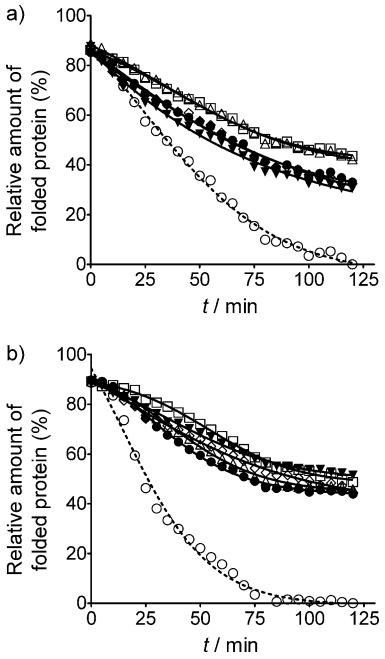


Figure 1. Thermal denaturation profile of Bor1 protein purified in DDM and then exchanged into novel XMAs (XMA-1—5) at two different detergent concentrations: a) CMC + 0.04 wt%, and b) CMC + 0.2 wt%. Bor1 in DDM at the two different concentrations was used as the control. Thermal stability of Bor1 protein was monitored by CPM assay performed at 40 °C for 120 min. The relative amounts of folded protein were normalized relative to the most destabilising condition in this experiment, that is, protein denaturation in DDM after 2 h incubation. The data are representative of three independent experiments. ○: DDM; △: XMA-1; ▼: XMA-2; ◇: XMA-3; ●: XMA-4; □: XMA-5.

by SDS-PAGE and Western blotting analysis. As shown previously,[13e,g,15e] the conventional detergent, DDM, completely extracts $MelB_{St}$ from membranes, yielding the highest solubilisation at both 0 °C and 45 °C, but little or no protein was observed when solubilised at 55 or 65 °C, respectively (Figure 2). This result indicates that the DDM-solubilized $MelB_{St}$ tends to denature/aggregate with increasing temperature. $MelB_{St}$ solubilisation in the XMAs at 0 °C yielded a reduced amount of the protein (Figure 2 and Figure S2a in the Supporting Information). At 45 °C, however, the amount of soluble $MelB_{St}$ increased from less than 15% up to 50% with XMA-1 and 80% with XMA-5, and from 50% up to 70% with XMA-2 and 90% with XMA-3. The temperature effect indicates that the reduced amount of soluble $MelB_{St}$ with each of these new agents at 0 °C is due to poor solubilisation efficiency of these detergents but not due to aggregation/denaturation of $MelB_{St}$ protein. When the solubilisation temperature was further increased to 55 °C, the amounts of $MelB_{St}$ solubilized by the XMAs decreased; however, a significant amount of soluble $MelB_{St}$ was obtained with XMA-2, XMA-3, or XMA-5. It is noteworthy that, even at 65 °C, there are still small amounts of $MelB_{St}$ detectable following extraction with XMA-3 and XMA-5, in contrast to the complete absence of protein solubilised with DDM at this temperature. Overall, these results indicate that XMA-2, XMA-3 and XMA-5 are superior to DDM in stabilising $MelB_{St}$ although the solubilisation efficiency achieved by DDM is higher. Of note,

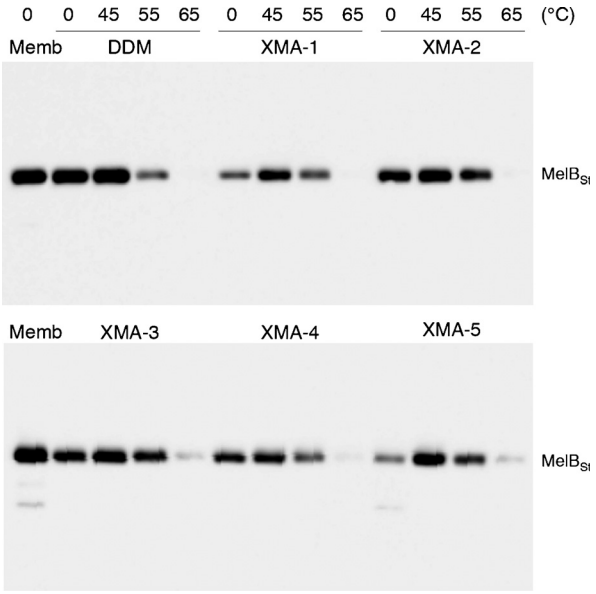


Figure 2. SDS-PAGE and Western blot analysis of MelB$_{St}$. Identical amounts of membrane containing MelB$_{St}$ were treated with the individual detergents (XMA-1–5, and DDM) at 1.5 wt% for 90 min at the specified temperatures, and the samples were analysed by SDS (16%)-PAGE following ultracentrifugation. The amount of soluble protein was detected using Western blotting with anti-His tag antibody. An untreated membrane sample ("Memb") was included as a control.

protein solubilisation could be increased by a longer incubation time. For example, we found a substantial increase in solubilisation efficiency of the novel agents when the experiment was carried out at 4 °C, overnight (Figure S2).

These promising results of the novel agents for membrane protein stabilisation prompted us to test them with human β_2 adrenergic receptor (β_2AR), a G-protein coupled receptor (GPCR).[21] In order to explore the effect of the novel agents on the conformational change of β_2AR, we measured fluorescence changes in a bimane fluorophore associated with alterations in receptor conformation upon ligand and G-protein binding.[22] The bimane moiety is covalently attached to cysteine 265 located at the cytoplasmic end of transmembrane helix 6 (TM6). Thus, receptor conformation as well as conformational changes associated with inactive and active states of the receptor could be precisely detected by the changes of fluorescence emission spectrum of the monobromobimane-labelled β_2AR (mBBr-β_2AR).[23] For this experiment, DDM-purified mBBr-β_2AR at 1 mg mL^{-1} was diluted into individual detergent solutions at a concentration of CMC + 0.04 wt%, and the bimane fluorescence spectra were recorded in the absence or presence of a high-affinity agonist, BI-167107 (Figure S3 in the Supporting Information). Of the five new agents, XMA-4 and XMA-5 resulted in bimane spectra similar to that of DDM, indicating effective preservation of the receptor activity by these two XMAs. Binding of a full agonist (e.g., BI) to the receptor is known to be insufficient to fully activate the receptor, which further requires G-protein binding.[14a] A similar result was found in this study for mBBr-β_2AR in the presence of the full agonist, isoproterenol (ISO), although slight differences in the bimane spectra between DDM and XMA-4- or XMA-5-solubilised protein were observed (Figure 3a and Figure S4 in the Supporting Information). The ability of β_2AR solubilised in XMA-4 or XMA-5 to properly activate G-protein was characterized by G-protein-coupling assay.[14a] As can be seen in Figure 3a, the bimane spectra of XMA-4-solubilized receptor–G-protein complexes are similar to that of the DDM-solubilized complex. A similar trend was observed using XMA-5-solubilized receptor (Figure S4). The results indicate that these two agents behave well for receptor activation by agonist binding and G-protein coupling. The reduction in fluorescence intensity and the shift in maximal emission wavelength observed is ascribed to conformational changes of β_2AR associated with the transition from the inactive to active state caused by the binding of both ISO and G-protein.[14a,n] Detergent efficacy (XMAs vs. DDM) was further compared by diluting these agents far below their respective CMC values. As shown in Figure 3b, DDM-solubilized β_2AR underwent an obvious conformational change by this dilution while XMA-4- and XMA-5-solubilized receptors underwent only minor changes, suggesting a slow off-rate for these new agents from the receptor compared to DDM (Figure S5 in the Supporting Information). These intriguing results prompted us to carry out the ligand-binding assay for β_2AR after detergent exchange. The receptor activity purified in DDM and XMAs were assessed by binding of [^{3}H]dihydroalprenolol ([^{3}H]DHA). The receptor purified in both XMA-4 and XMA-5 showed a level of radioligand binding affinity similar to DDM-purified receptor, indicating that these two XMAs could be useful alternatives to DDM, the best conventional detergent for β_2AR study. Next, we moved to the leucine transporter (LeuT) from *Aquifex aeolicus*,[24] for the evaluation of the novel agents to stabilise a secondary active transporter. Protein activity in aqueous solutions supplemented with individual XMAs or DDM was measured by a scintillation proximity assay (SPA) at regular intervals over 12 day incubation period at room temperature.[25] DDM-purified transporter at 1.5 mg mL^{-1} was used as a stock solution. At a detergent concentration of CMC + 0.04 wt%, all XMAs were inferior to DDM (Figure S6a in the Supporting Information). When we increased detergent concentration to CMC + 0.2 wt%, only XMA-4 and XMA-5 showed comparable efficacy to DDM only in the latter part of the incubation period (i.e., from day 5 to 12), indicating that most XMAs have limited stabilising effect on this particular membrane protein as compared to DDM (Figure S6b).

Detergent efficacy is known to be membrane protein specific. When we evaluated XMAs for four membrane proteins systems and compared with DDM, the most common conventional detergent for membrane protein study,[26] in terms of membrane protein stabilisation efficacy, some new agents appeared to be superior to DDM for Bor1 and MelB$_{St}$ while those agents were comparable to DDM for β_2AR and inferior to DDM for LeuT. Of the XMAs, XMA-4 and XMA-5 showed generally favourable behaviour for protein stability; even for LeuT, these agents are the most comparable to DDM. We believe that these agents with C11 and C12 alkyl chains, respectively, are the most hydrophobic of XMAs and thus have optimal hydrophile–lipophile balance (HLB). In contrast, XMA-

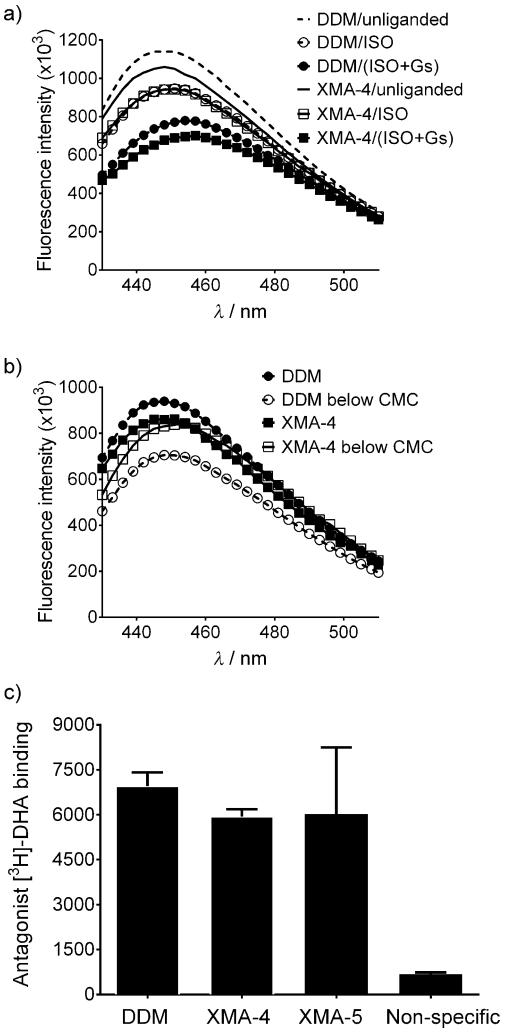


Figure 3. Fluorescence spectra of: a) monobromobimane-labelled β_2AR (mBBr-β_2AR) solubilized in DDM and XMA-4 in the absence or presence of full agonist (isopreoterenol (ISO)) and a combination of ISO and G-protein (Gs), and b) those of unliganded mBBr-β_2AR at detergent concentrations below their respective CMC values. DDM- or XMA-4-solubilized receptor was diluted 1000-fold into an aqueous buffer with no detergents. The data are representative of three independent experiments. c) Ligand binding activity for β_2AR solubilized in DDM or novel amphiphiles (XMA-4 and XMA-5). The protein activity was measured with the radioligand-binding assay using the antagonist [^{3}H]dihydroalprenolol (DHA). Detergents were used at CMC + 0.04 wt% for these evaluations.

1 with the shortest alkyl chain is likely to be much more hydrophilic, explaining why in most cases this produces the least stable protein. Detergent HLB number has been shown to play a critical role in detergent behaviour.[27] Of note, XMAs have a distinct architecture from conventional detergents, but can be prepared in just five high-yielding synthetic steps, making those agents highly accessible. In addition some XMAs, particularly XMA-4 and XMA-5, conferred greater stability to a range of membrane proteins, suggesting that these agents could be good alternatives to conventional detergents for membrane protein study.

Many important questions need to be addressed in the next study. For example, it would be valuable to determine micellar aggregation numbers formed by XMAs and how these numbers are affected by the chain length of the novel agents. More importantly, XMAs displayed favourable behaviours for protein stabilisation efficacy, but these agents were suboptimal for LeuT. It is impossible to provide a precise reason for this limited behaviour, but it may be related to the long distance between the two alkyl chains of XMAs. Because of this structural feature detergent packing around the membrane protein may not be tight enough, leading to a decrease in both the detergent hydrophobic density and detergent affinity for the membrane protein. Currently we are developing new amphiphiles with increased hydrophobic density to address this topic. However, we believe that, based on the first characterization shown here, some XMAs will be useful tools for membrane protein study.

Experimental Section

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

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