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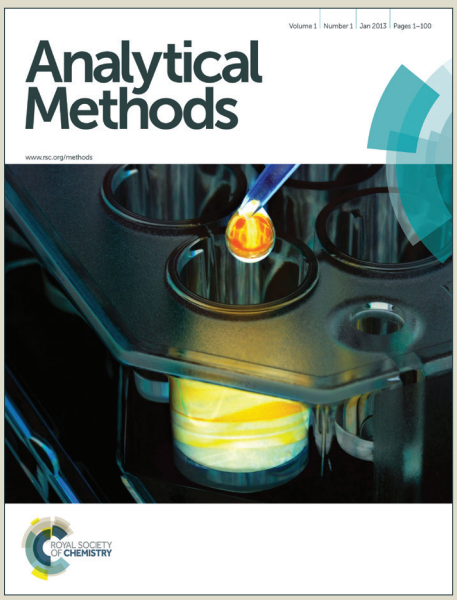

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Accessible glyco-tripod amphiphiles for membrane protein analysis

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Membrane protein manipulation is known to be an extremely challenging task, mainly because of incompatibility between the hydrophobic surface area of proteins and the hydrophilic character of an aqueous medium. To avoid protein degradation resulting from this incompatibility, detergents are essential tools in the study of membrane proteins. However, traditional detergents have a limited ability to stabilize the native conformation of membrane proteins. This study introduces a novel tripod amphiphile that can be prepared efficiently from a commercially available compound. The new agent proved very effective for the long-term stability of a multi-subunit superassembly, a membrane protein sensitive to denaturation.

Introduction

Integral membrane proteins are essential components of lipid membranes for cellular activity. These bio-macromolecules constitute approximately 50% of membranes by mass and carry out central functions such as material transfer, signal transduction, and cell-to-cell communications. The importance of these macromolecules can be well illustrated by the fact that more than 50% of current pharmaceutics target membrane proteins.[1] Despite such importance in biology and drug discovery, the structural and functional information on membrane proteins is very limited relative to their soluble counterparts. Membrane proteins account for fewer than 1% of total proteins with known structure and the precise mechanism of actions of many membrane proteins are still unknown, indicating the extreme difficulty of membrane protein structural and functional studies.[2] This difficulty is attributed to the inherent instability of membrane proteins.[3] Conventional detergents such as *n*–octyl–β–D–glucopyranoside (OG), *n*–dodecyl–β–D–maltoside (DDM), and lauryldimethylamine–*N*–oxide (LDAO) are widely used in membrane protein research to keep the proteins soluble and functional.[4] As a result of hydrophobic interactions, these detergents tend to form self-assemblies with a hydrophobic interior and a hydrophilic exterior called micelles in aqueous media.[5] Thus, these amphipathic agents have the ability to associate with the hydrophobic surface of membrane proteins, leading to the production of protein–detergent complexes (PDCs).[6] However, when encapsulated by these agents many membrane proteins have a tendency to undergo structural degradation, precluding the chance to obtain protein crystals suitable for structure determination.[7] Thus, it is of great interest to develop novel amphiphiles highly effective for stabilizing isolated membrane proteins .

Over the last two decades, a number of novel agents have been developed. Some of these agents were conveniently prepared by simple modifications of conventional detergents as exemplified by Chae's Glyco–Tritons (CGTs)[8a] and cholate– or deoxycholate–based *N*–oxides (CAOs or DCAOs).[8b] Others were invented by introducing new hydrophobic groups into detergent scaffolds. Examples include hemifluorinated surfactants (HFSs), chobimalt, glyco-diosgenin (GDN), and adamantane-based amphiphiles (ADAs).[9a-d] HFSs contain fluorinated alkyl chains as detergent hydrophobic groups[9a] whereas chobimalt, GDN, and ADAs utilize very rigid hydrophobic groups: cholesterol, diosgenin and adamantane, respectively.[9b,c,d] The secondary structures of peptides have often been employed to generate new amphiphiles by taking advantage of convenient peptide synthesis. Lipopeptide detergents (LPDs) with α–helical structure,[10a] short peptides,[10b] and β-peptides (BPs)[10c] are representative examples of these peptide-based novel agents. Innovative approaches made it possible to invent polymeric materials such as amphipols (APols),[11a,b] nanolipodisq,[11c] and nanodiscs (NDs)[11d] that are quite different from the conventional architecture with a single head group and a single alkyl chain. These materials displayed favorable behavior, particularly for membrane protein stabilization, although they have not been shown to be efficient for membrane protein solubilization. These peptide-based agents and polymeric materials are yet to contribute to high-resolution membrane protein structures.

Among the novel classes of amphiphiles developed so far, facial amphiphiles (FAs)[12a,b] and neopentyl glycol (NG) agents (glucose neopentyl glycols [GNGs]),[12c,d] and maltose neopentyl glycols [MNGs][12e-g]) have been the most successful for membrane protein structural studies. FAs have enabled determination of the crystal structures of a few membrane proteins[12a] and NG class amphiphiles have facilitated about 20 new crystal structure determinations of versatile membrane proteins, including several G-protein coupled receptors (GPCRs) and their G-protein complexes.[13a-j] On the other hand, tripod amphiphiles (TPAs) are probably the most extensively studied of the small novel agents.[14a-f] Initially, *N*–oxide versions of

this class (*e.g.*, TRIPAO, which is commercially available) were developed[14a], but the rather strong nature of these agents with respect to protein denaturation prompted us to prepare TPAs with a carbohydrate head group (*e.g.*, a maltoside or a branched diglucoside).[14b] When we evaluated a number of branched diglucoside–bearing TPAs for membrane protein stability, most of these agents were shown to be effective at stabilizing a denaturation–prone membrane protein complex. In contrast, most maltose-bearing TPAs showed a rather harsh nature regarding membrane protein stability.[14b-d] One weakness of these glyco-TPAs is their synthetic inconvenience because seven synthetic steps are required to give each amphipathic compound.[14e] Thus, the purpose of this study was to introduce a new branched diglucoside-bearing TPA with synthetic convenience, thereby substantially enhancing its availability. We evaluated this agent, designated TPA-18, with a multi-subunit, pigment protein complex in terms of protein extraction from the membrane and long-term protein stability.

Results and Discussion

We prepared the new agent in a straightforward synthetic protocol. Starting from a carboxylic acid derivative with three phenyl groups, we could prepare the new TPA via three synthetic steps, comprised of amide coupling, glycosylation, and deprotection (**Scheme 1**). The overall yield of these three synthetic steps is ~ 80%, which allows us to prepare this agent on a kilogram scale without any issues (see experimental section for details). Previously reported TPAs require at least six steps for their synthesis, comprising Knoevenagel condensation, Michael addition of an alkylidene compound, basic hydrolysis of a dinitrile derivative, amide coupling, glycosylation and deprotection (**Scheme S1**).[14a] Furthermore, Michael reaction using a higher order cuprate is sensitive to moisture and typically gave a low yield (~60%). A very high temperature along with a long reaction time (200 °C and three days) was used to hydrolyze of a dinitrile derivative under the basic condition. This multi-step synthesis leads to overall yield of ~40%. Thus, the synthetic efficiency for the preparation of TPA-18 is the best among all glyco-TPAs synthesized to date. Solubility tests showed that the new agent is water-soluble at more than 20 wt%. This high solubility is ascribed to the polar nature of a phenyl group relative to an aliphatic alkyl chain with the same carbon unit (C6) and the high hydrophilicity of the branched diglucoside head group (**Figure 1**); this head group is more hydrophilic than maltoside group, giving better solubility in aqueous media.

The critical micelle concentration (CMC) and micelle size of the agent were estimated using a fluorescent probe, diphenylhexatriene (DPH)[15] and dynamic light scattering (DLS), respectively. The results are summarized, together with those for some previously reported TPAs (TPA-2, TPA-8, and TPA-15) and conventional detergents (DDM and LDAO), in **Table 1**. The new agent gave a rather large CMC value, probably because of the presence of the three large lipophilic groups. Large hydrophobic group-bearing detergents tend to show high CMC values because steric congestion inside the detergent micelles disfavors micelle formation.[16] Detergent CMC values could vary depending on buffer composition. Accordingly, the CMC value of the new agent was estimated under the conditions of three different pHs (7.0, 7.5 and 8.0) and three different salt concentrations (0 mM, 100 mM and 200 mM) (**Table S1**). We found that the CMC value of the new agent was almost invariable with pH change from pH 7.0 to pH 8.0. However, when salt concentration was increased from 0 mM to 200 mM, its CMC value was slightly changed from ~ 8.3 mM to ~ 7.6 mM. These results are consistent with the general notion that the CMC value of a nonionic detergent such as OG or DDM is relatively independent on the pH and salt concentration of a buffer solution. Micelle size formed by the new agent was comparable to those of TPA-2 and TPA-15 (~2.2 nm), but was relatively small when compared with that of TPA-8 (~7.1 nm). It is notable that TPA-18 has a CMC value and micelle size quite different from those of TPA-8 despite the fact that they have detergent hydrophobic groups with the same number of carbons (C20). This result indicates that the aggregation tendency and micelle size of a detergent are strongly dependent on their overall architecture, particularly the volume of the detergent hydrophobic groups. Introduction of a bulky group into detergent lipophilic region decreased the micelle size and aggregation tendency (TPA-8 *vs.* TPA-18; **Table 1**). In a previous study, detergents with branched hydrophobic groups showed a tendency to form large micelles with increasing detergent concentration.[17] A similar trend was observed for three TPAs (TPA-2, TPA-8, and TPA-18; **Table 2** & **Figure S1 and S2**). All three TPAs containing branched tail groups showed enlarged micelles when we increased the detergent concentration from 1.0 wt% to 2.0 wt% to 4.0 wt%. Such concentration dependency was most prominent in TPA-8 and least obvious in TPA-2 (**Figure S2**). In contrast, DDM with a straight alkyl chain showed no significant variation in micelle size when detergent concentration was changed in the same range.

Table 1. Critical micelle concentrations (CMCs) and hydrodynamic radii (R_h) of micelles for previously reported TPAs (TPA-2, TPA-8, and TPA-15), a newly reported TPA (TPA-18), and three conventional detergents (LDAO, OG, and DDM). All detergents were dissolved in pure water.

	MW[a]	CMC (mM)	CMC (wt%)	R_h (nm)[b]
TPA-2	659.8	~3.6	~0.24	2.2 ± 0.1
TPA-8	715.9	~0.42	~0.030	7.1 ± 0.1
TPA-15	649.8	~4.7	~0.31	2.2 ± 0.0
TPA-18	699.7	~8.3	~0.58	2.2 ± 0.0
OG	292.4	~25	~0.73	1.5-2.3[c]
LDAO	229.4	1-2	~0.023	2.0[d]
DDM	510.1	~0.17	~0.0087	3.5 ± 0.03

[a]Molecular weight of detergents. [b]Hydrodynamic radius of micelles determined at 2.0 wt% by dynamic light scattering. [c,d]Values obtained from literatures.[18a,b]

Table 2. Changes in hydrodynamic radii of detergent micelles (mean ± SD, n = 4) according to detergent concentration for three branched TPAs (TPA-2, TPA-8, and TPA-18) and a linear detergent (DDM).

Concentration	1.0 wt%	2.0 wt%	4.0 wt%
TPA-2[a]	2.0 ± 0.06	2.2 ± 0.05	2.3 ± 0.02
TPA-8	5.6 ± 0.05	7.1 ± 0.10	8.7 ± 0.11
TPA-18	1.8 ± 0.03	2.2 ± 0.01	2.4 ± 0.02
DDM[a]	3.5 ± 0.03	3.5 ± 0.03	3.6 ± 0.02

[a]Values obtained from a literature. [17]

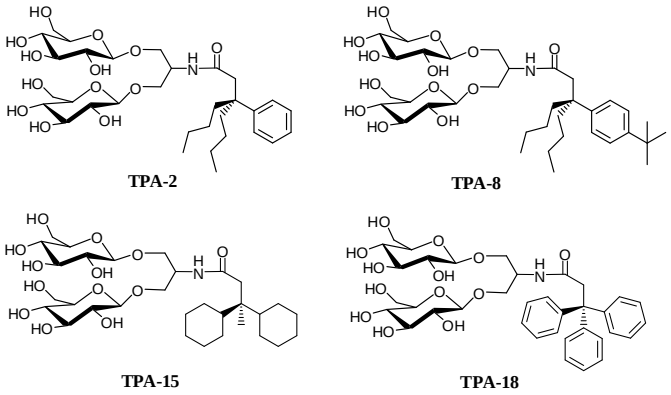


Figure 1. Chemical structures of previously reported TPAs (TPA-2, TPA-8, and TPA-15) and a newly reported TPA (TPA-18).

The photosynthetic superassembly of *R. capsulatus*, comprised of a light harvesting complex I (LHI) and reaction center complex (RC),[19] was used to evaluate the performance of the new TPA (TPA-18). For comparison, previously reported TPAs (TPA-2, TPA-8, and TPA-15) were included, together with three conventional detergents (OG, LDAO, and DDM). Because the superassembly contains multiple cofactors such as chlorophylls and carotenoids, this protein gives rise to a characteristic UV-visible spectrum, which can be used to detect any changes in the conformation of detergent-solubilized proteins over the course of long-term incubation. First, LHI-RC complexes were solubilized and purified in a well-behaved conventional detergent (DDM) and the DDM-purified protein was then diluted into buffer solutions containing individual TPAs or conventional detergents at the designated concentrations. The final concentration of DDM was far lower than its CMC value because of the high dilution factor; the CMC of DDM was ~ 0.0087 wt% and the final DDM concentration following dilution is about 0.00044 wt%. Protein samples solubilized in individual detergents were incubated at room temperature under dark conditions and their UV-visible spectra were recorded over 20 days to assess the long-term protein stabilization of each detergent. The absorbance values of protein samples at 875 nm (A_{875}) were used to assess protein integrity as a function of time because the superassembly with the native conformation produces an intense peak at this wavelength whereas a partially denatured protein complex shows a reduction in the 875-nm peak intensity and concomitant appearance of new peaks at ~760 nm and/or 800 nm. To investigate the effect of detergent concentration on protein stability, three detergent concentrations were used: CMC+0.04 wt% (low), CMC+0.2 wt% (medium) and CMC+1.0 wt% (high). As shown in **Figure 2**, when detergents were evaluated at CMC+0.04 wt%, DDM was the most promising among the conventional agents for retaining the native conformation of LHI-RC complexes, consistent with the widespread use of this detergent for membrane protein studies.[20] A zwitterionic detergent (LDAO) was the worst, giving complete degradation in a couple of days, whereas a nonionic detergent (OG) was better than LDAO, but still showed a strong propensity to degrade the native protein structure. These results of conventional detergents are in good agreement with our general knowledge of detergent behavior.[4] As expected, all tested TPAs (TPA-2, TPA-8, and TPA-15) were more favorable than or comparable to DDM for stabilization of LHI-RC complexes. Consistent with a previous study, TPA-2 and TPA-15 were superior to TPA-8 in this regard, giving only 10-20% degradation after 20 days of incubation.[14d] The new agent, TPA-18, was even better than these successful predecessors. When we increased the detergent concentration to CMC+0.2 wt% and CMC+1.0 wt%, overall detergent efficacies were worse than those observed at the low concentration of CMC+0.04 wt%. These results are consistent with a previous observation; excess detergent micelles exacerbate protein denaturation.[9a] Importantly, the difference in detergent efficacy for membrane protein stabilization increased with detergent concentration, leading to the largest difference at the high detergent concentration (CMC+1.0 wt%). Thus, the superior behavior of TPA-18 for superassembly stability could be clearly observed under this condition. Notably, the stabilizing effect of TPA-18 at CMC+1.0 wt% (high concentration) was even better than that of DDM at CMC+0.04 wt% (low concentration), indicative of the very mild nature of TPA-18 micelles compared to the micelles of the other tested TPAs and DDM.

High detergent concentrations (e.g., CMC+1.0 wt%) are typically employed to solubilize a target membrane protein from the native membrane. Thus, this result may indicate that TPA-18 has the ability to extract membrane proteins from membranes without destroying protein integrity. When we evaluated this agent for the solubilization of LHI-RC complexes, TPA-18 could indeed extract LHI-RC complexes from the membrane without structural degradation although its solubilization efficiency was not high (~40%; see supporting **Figure S3**). The weak intensity observed for the spectrum of TPA-18-solubilized LHI-RC complexes relative to those of the other tested detergents is due to its low protein solubilization efficiency (**Figure S3**). Note that protein solubilization yield could be increased by changing solubilization conditions (e.g., detergent concentration, assay temperature, or incubation time with a detergent). We did not carry out such an optimization process in this study.

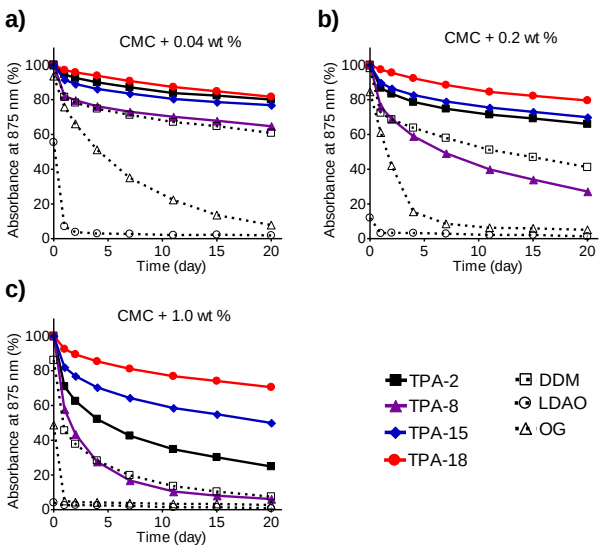
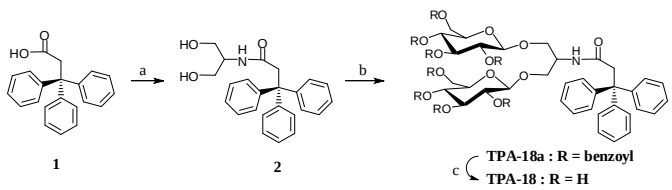


Figure 2. Long-term stability profile of *R. capsulatus* superassembly purified in conventional detergents (DDM, OG, and LDAO), previously reported TPAs (TPA-2, TPA-8, and TPA-15), and a newly reported TPA (TPA-18). The stability of the superassembly was monitored over 20 days by measuring absorbance value at 875 nm (A_{875}). For these measurements, detergent-purified protein samples were incubated at room temperature.

At this point, it is not clear why TPA-18 showed superior behavior in membrane protein stabilization compared to other glyco-tripod agents. The presence of multiple phenyl groups in the lipophilic region may enable the formation of favorable intermolecular interactions (*e.g.*, π-π stacking) between detergent molecules surrounding a target membrane protein. Such interactions could make the protein-surrounding detergent micelles more robust, and therefore better at protecting the target membrane protein from an aqueous medium. In the case of the other tested TPAs with a single phenyl group (TPA-2 and TPA-8), the presence of alkyl chains (*e.g.*, butyl chains) may hinder intermolecular interactions between the phenyl groups.

TPAs were initially developed with the intent to increase detergent rigidity because conformational rigidity might facilitate membrane protein crystallization, which necessitates an ordered state.[14e] This hypothesis was supported by the successful outcomes of TRIPAO in the crystallization of bacteriorhodopsin (BR) and bovine rhodopsin (rho) proteins.[14e] In this context, TPA-18 is considered to be the most rigid amongst TPAs developed so far because it has three phenyl groups without an alkyl chain. Additionally, its characteristic of forming small micelles could be beneficial for membrane protein crystallization because the PDCs formed by this agent would be small, which makes a large hydrophilic protein surface area available for protein crystal lattice formation.[21] TPA-18 forms even smaller micelles than TPA-2; the micelle size of TPA-18 is comparable to those of LDAO and OG, two conventional detergents that are popular for membrane protein structural studies. One weakness of TPA-18 is the relatively low membrane protein solubilization efficiency. This low efficiency observed for TPA-18 may be overcome by optimizing protein solubilization conditions (e.g., by increasing incubaton temperature and/or incubation time). Alternatively, the protein solubilization efficiency could be improved by increasing detergent hydrophobicity (*e.g.*, by introducing an alkyl chain on a benzene ring). However, a new strategy may be necessary to achieve such structural modifications without interfering with favorable intermolecular interactions between the detergent hydrophobic groups (*e.g.*, π-π stacking).

Experimental Section



Scheme 1. Synthetic scheme of TPA-18. (a) Serinol, EDC • HCl, HOBt, DMF, room temperature; (b) perbenzoylated glucosylbromide, AgOTf, CH_2Cl_2, -45°C → 0°C; (c) NaOMe, MeOH, room temperature.

The carboxylic acid derivative (**1**) (3.8 mmol), serinol (7.6 mmol), and 1-hydroxybenzotriazole monohydrate (HOBt • H_2O) (1.2 g, 9.1 mmol) were dissolved in anhydrous DMF (30 mL). 1-(3-(dimethylamino)propyl)-3-ethylcarbodiimide hydrochloride (EDC • HCl) (1.7 g, 9.1 mmol) was then added in small portions at 0°C, and the resulting solution was stirred at room temperature for 40 h. The solution was taken up with EtOAc (100 mL) and washed successively with a 1 M aqueous $NaHCO_3$ solution (100 mL), a 0.1 M aqueous HCl solution (100 mL), and brine (2 × 100 mL). The organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed by rotary evaporation. Column chromatography (EtOAc/hexane) afforded the desired alcohol derivative (**2**) as a solid in 92% yield. **^{1}H NMR** (300 MHz, $CDCl_3$+10% CD_3OD): δ 7.33-7.11 (m, 15H), 6.20 (d, *J* = 8.2 Hz, 1H), 4.28 (br s, 2H), 3.57 (s, 3H), 3.40-3.29 (m, 2H), 3.25-3.13 (m, 2H); **^{13}C NMR** (75 MHz, $CDCl_3$+10% CD_3OD): δ 171.4, 146.4, 129.2, 127.9, 126.3, 60.8, 56.3, 52.2, 47.9; **HRMS (ESI)**: calcd. for $C_{24}H_{25}NO_3$ $[M+H]^+$ 376.1908, found 376.1909.

TPA-18a was prepared at 90% yield according to the method in the literature[22] with slight modification. A mixture of alcohol derivative (**2**), AgOTf (2.4 equiv.), and 2,4,6-collidine (2.0 equiv.) in anhydrous CH_2Cl_2 (40 mL) was stirred at -45°C. A solution of perbenzoylated glucosylbromide (2.4 equiv.) in CH_2Cl_2 (40 mL) was added to this suspension over 10 min. Stirring was continued for 0.5 h at -45°C, and then the reaction mixture was allowed to warm to 0°C and stirred for a further 1 h. After completion of the reaction (as detected by TLC), pyridine was added to the reaction mixture, which was diluted with CH_2Cl_2 (40 mL) before being filtered through a pad of celite. The filtrate was washed successively with 1 M aqueous $Na_2S_2O_3$ solution (40 mL), 0.1 M aqueous HCl solution (40 mL), and brine (2 × 40 mL). The organic layer was dried with anhydrous Na_2SO_4, and the solvent was removed by rotary evaporation. The residue was purified by silica gel column chromatography (EtOAc/hexane), which provided the desired product as a glassy solid. **^{1}H NMR** (300 MHz, $CDCl_3$): δ 8.18-8.07 (m, 4H), 8.05-7.97 (m, 4H), 7.96-7.88 (m, 4H), 7.87-7.78 (m, 4H), 7.74-7.59 (m, 6H), 7.59-7.48 (m, 4H), 7.48-7.35 (m, 10H), 7.35-7.19 (m, 17H), 7.19-7.08 (m, 3H), 5.60-5.42 (m, 4H), 5.34-5.18 (m, 3H), 4.58-4.39 (m, 2H), 4.39-4.25 (m, 2H), 4.00-3.85 (m, 1H), 3.75-3.62 (m, 2H), 3.60-3.49 (m, 1H), 3.49-3.32 (m, 3H), 3.32-3.21 (m, 2H), 3.21-3.09 (m, 1H), 2.55 (t, *J* = 9.5 Hz, 1H); **^{13}C NMR** (75 MHz, $CDCl_3$): δ 169.9, 166.2, 166.0, 165.9, 165.3, 165.2, 164.9, 164.8, 146.5, 134.0, 133.9, 133.7, 133.6, 133.5, 133.4, 130.2, 129.9, 129.8, 129.7, 129.4, 129.1, 129.0, 128.7, 128.6, 128.5, 128.2, 126.4, 101.5, 101.3, 72.6, 72.1, 71.9, 71.8, 69.7, 69.6, 67.5, 66.5, 63.2, 63.1, 56.5, 48.5, 47.1; **MS (MALDI-TOF)**: calcd. for $C_{92}H_{77}NO_{21}Na[M+Na]^+$ 1554.6, found 1554.9.

TPA-18 was prepared at 93% yield by de-*O*-benzoylation under Zemplén's conditions.[22] TPA-18a was dissolved in MeOH and then treated with a catalytic amount of NaOMe such that the final concentration of NaOMe was approximately 0.05 M. The reaction mixture was stirred for 6 h at room temperature, and then neutralized with Amberlite IR-120 (H^+ form) resin. The resin was removed by filtration and washed with MeOH, and solvent was removed from the combined filtrate by rotary evaporation. The residue was purified by silica gel column chromatography (MeOH/CH_2Cl_2). Further purification by recrystallization using CH_2Cl_2/MeOH/diethyl ether afforded fully deprotected product as a white solid. **^{1}H NMR** (300 MHz, CD_3OD): δ 7.31-7.13 (m, 15H), 4.13 (dd, *J* = 7.5, 4.5 Hz, 2H), 3.95-3.82 (m, 3H), 3.77-3.471 (m, 8H), 3.36-3.2.0 (m, 4H), 3.16-3.10 (m, 2H); **^{13}C NMR** (75 MHz, CD_3OD): δ 173.0, 148.5, 130.8, 128.9, 127.3, 104.9, 104.7, 78.1, 75.2, 75.1, 71.7, 69.3, 62.8, 57.8; **HRMS (ESI)**: calcd. for $C_{36}H_{45}NO_{13}$ $[M+H]^+$ 700.2964, found 700.2955.

Conclusion

A new accessible glyco-TPA (TPA-18) was efficiently prepared from a commercially available material and evaluated with LHI-RC complexes. We found that the new agent was clearly superior to three conventional detergents (DDM, OG, and LDAO) as well as three previously reported glyco-TPAs (TPA-2, TPA-8, and TPA-15) in maintaining the native structure of the membrane protein complex for long period of time. In addition, TPA-18 is highly rigid and forms small micelles. Therefore, this agent has a significant potential for membrane protein structural and functional study.

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Notes and references

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Electronic Supplementary Information (ESI) available: assay protocol for membrane protein solubilization and purification: Details may be found in the Supporting Information.

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