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Glycotripod Amphiphiles for Solubilization and Stabilization of a Membrane-Protein Superassembly: Importance of Branching in the Hydrophilic Portion

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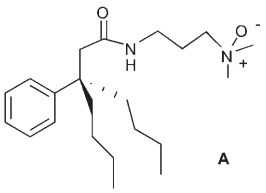
Isolation and physical characterization of membrane proteins remains a central challenge in biomolecular science.[1] Three-dimensional structure determination for membrane proteins, for example, has been successful only within the past two decades, and the set of known membrane-protein structures is far smaller than the set of known soluble-protein structures.[2] Synthetic amphiphiles, such as detergents, are crucial tools in this field: they are used to extract embedded proteins from the membranes in which they naturally occur and maintain native protein conformation in the solubilized state.[3] Physical characterization is often carried out with protein–amphiphile complexes, and such complexes are usually the basis for crystallization efforts; growth of high-quality crystals is a rate-limiting step in structure determination.[4] In light of the central role played by amphiphiles in membrane-protein science, surprisingly little effort has been devoted to exploration of non-traditional architectures for these small molecules.[5] Here, we describe new synthetic amphiphiles that display favorable solubilization and stabilization properties in a challenging biochemical system. The results reveal new principles for the design of membrane-protein solubilization agents.



A

We previously introduced "tripod amphiphiles", such as **A**,[6] which were intended to meet the need for new types of synthetic agents that could be used in place of standard detergents for membrane-protein manipulation.[7] Most detergents feature a lipophilic segment that is very flexible. This property can facilitate membrane-protein solubilization by allowing detergent molecules to accommodate themselves to lipophilic protein surfaces. It is possible, however, that detergent flexibility could discourage crystallization of the protein–detergent complex by allowing alternative detergent conformational states, which would disturb regular packing in a crystal lattice. A balance between flexibility and rigidity is presumably necessary for maximum utility. The branch-point in **A** imposes partial conformational restriction on the lipophilic segment because torsional motions are limited for bonds near the tetrasubstituted carbon.[8]

The new amphiphiles presented here feature carbohydrate-derived hydrophilic groups, and they include branch-points in the hydrophilic group as well as the lipophilic group. Our results show that branching in the hydrophilic portion of an amphiphile can be beneficial for extraction of an intact protein superassembly from the native membrane, whether the lipophilic portion is branched or linear. Moreover, we find synergy between branching in the lipophilic and hydrophilic portions of the amphiphile. Our experiments initially focused on glycotripod series TPA-1 to TPA-5 (Scheme 1), each of which contain the lipophilic tripod found in **A**. Glucosides and maltosides are prominent among membrane-protein detergents, and our series includes simple glucoside and maltoside derivatives (TPA-1 and TPA-4, respectively). In addition, we examined analogues that contain branched hydrophilic groups, diglucoside TPA-2, triglucoside TPA-3, and dimaltoside TPA-5. These new molecules were evaluated in terms of their ability to solubilize and stabilize membrane-protein complexes that comprise the functional core of the photosynthetic unit in *Rhodobacter* species of photosynthetic bacteria.[9]

The five glycotripod amphiphiles were prepared by using synthesis routes that readily provide multigram quantities.[6] These molecules displayed a considerable range of solubility and aggregation behavior in water. The monoglucoside TPA-1 was not water soluble and was not studied further. The other four glycotripod amphiphiles were highly soluble. Aqueous solubilization of Orange OT[10] was used to determine critical micelle concentrations (CMC). The values determined for TPA-2 (diglucoside) and TPA-4 (maltoside), 3.6 and 4.0 mM, respectively, are comparable to the CMC of **A** (5.5 mM).[6b] Neither TPA-3 nor TPA-5 solubilized Orange OT, which suggests that self-association of these amphiphiles is hindered by the hydrophilicity and/or steric bulk of the triglucoside and dimaltoside head-groups.

We used the transmembrane protein superassembly formed by the light harvesting-I (LHI) complex and the reaction center (RC) complex of the photosynthetic bacterium *R. capsulatus* to evaluate the efficacy of our new amphiphiles and to compare these amphiphiles with conventional detergents.[11] The LHI–RC superassembly represents a demanding system for solubilization and stabilization with a synthetic amphiphile. Each monomeric superassembly contains 12–18 LHI complexes, and each

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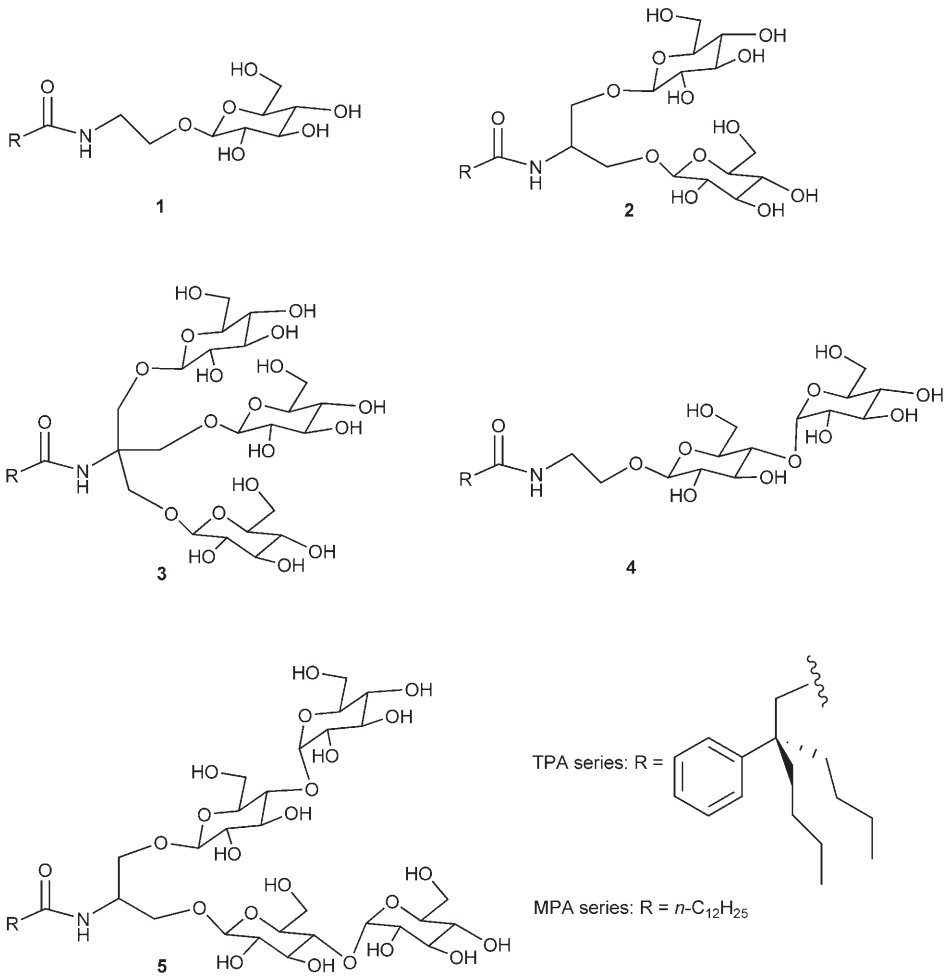
Supporting information for this article is available on the WWW under http://www.chembiochem.org or from the author: Synthesis and characterization of amphiphiles, detergent screening, and stabilization measurements.



Scheme 1. Chemical structures of tripod amphiphiles TPA-1 to TPA-5 and their monopod analogues MPA-1 to MPA-5.

LHI complex is comprised of two subunits. In addition, each monomeric superassembly contains a single copy of the RC complex, which is comprised of three subunits, and possibly other small polypeptides (e.g., PufX). Thus, to be successful an amphiphile must be aggressive enough to disrupt the native membrane but mild enough to preserve the tertiary structures of the (at least) five different proteins in the superassembly as well as the quarternary association of ~30–40 protein molecules. The cofactor constituents of the LHI–RC superassembly have unique spectral signatures in its native and denatured states, which facilitates amphiphile evaluation. A range of outcomes is possible in terms of solubilization (complete, partial, or none) and stabilization (no degradation, partial degradation, or complete degradation) over time. These various outcomes can be readily distinguished by steady-state spectroscopy.[9] Thus, graded comparative evaluations can be obtained easily for a set of candidates, such as TPA-2 to TPA-5. An ideal amphiphile will extract the intact LHI–RC superassembly from a bacterial membrane preparation and maintain the natural interactions among functional components. Amphiphiles with a more disruptive effect will dissociate and denature the very labile LHI complex to leave only intact RC, and even harsher amphiphiles will cause RC degradation. Each of these various outcomes can be assessed unambiguously by optical spectroscopy (Figure 1, inset).

Preliminary studies revealed that **A** is too harsh to be useful for the preparation of intact superassembly (Figure 1), because LHI is extensively denatured. The strong absorption near 760 nm after solubilization with **A** arises from bacteriopheophytins, that is, bacteriochlorophyll units that have dissociated from LHI complexes and lost the central Mg ion.[9d] TPA-3, on the other hand, is too mild to be useful, as no protein was extracted from the membrane with this amphiphile. This result is perhaps not surprising, given that TPA-3 does not solubilize Orange OT. TPA-5, too, failed to solubilize Orange OT, but TPA-5 nevertheless solubilized a small proportion of relatively intact LHI–RC superassembly (see the Supporting Information). TPA-4 extracts a significant proportion of the membrane-

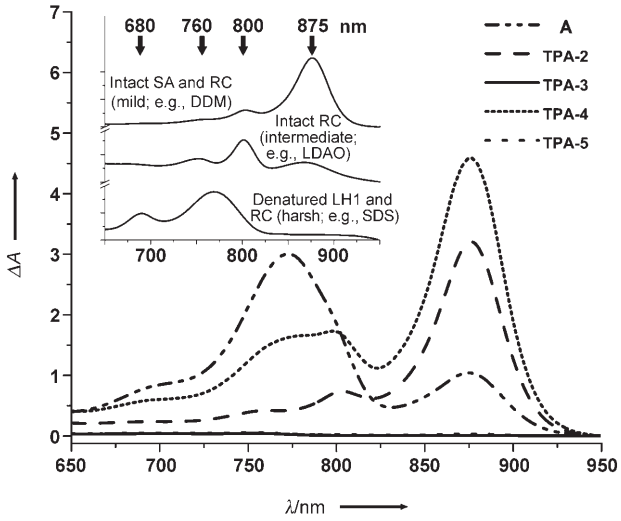


Figure 1. Spectroscopic comparison of solubilized protein complexes extracted from intracytoplasmic membranes of *R. capsulatus* by using glycotripod amphiphiles. In order to remain within the dynamic range of the spectrophotometer (OD < 1.5), we used diluted solutions for the more strongly absorbing samples. The absorbance spectra for the original samples, prior to dilution, were then calculated from the observed spectra by multiplication with the appropriate dilution factor. The harshness of the detergent can be judged by the intensity and features in the spectra, which represent linear combinations of the spectra of intact superassembly (SA), intact reaction center (RC), and denatured complexes (inset), as described in the text.

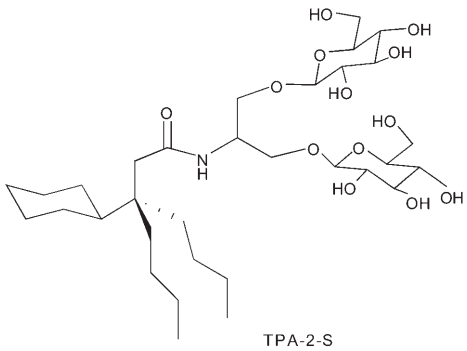
embedded protein, but the extracted protein includes a substantial amount of denatured LHI (absorbance in the 750–800 nm region); thus, TPA-4 is too harsh.

TPA-2 displayed excellent properties, in contrast to **A**, TPA-3, TPA-4, or TPA-5. Treatment of *R. capsulatus* membranes with TPA-2 provided intact LHI–RC superassembly in high yield (strong absorption at 875 nm; 875/760 nm absorption ratio ~8; Figure 1). It is noteworthy that TPA-2 and TPA-4 each have two glucose units in the hydrophilic segment and displayed similar CMC values, but that TPA-2 is clearly superior to TPA-4 with regard to extraction of intact photosynthetic superassembly from the native membrane. This functional difference suggests that incorporation of a branch-point in the hydrophilic portion leads to significantly improved performance relative to traditional hydrophilic group architectures.

Does hydrophilic group branching confer distinctive properties in the context of a more conventional lipophilic group? We explored this question by preparing monopod amphiphiles MPA-1 to MPA-5 (Scheme 1)—analogues of TPA-1 to TPA-5 in which the lipophilic tripod was replaced by a 12-carbon linear segment. MPA-1 and MPA-4 were not soluble in water. CMC values were determined by Orange OT solubilization for MPA-2 (2.4 mM), MPA-3 (4.4 mM), and MPA-5 (1.7 mM). MPA-3 and MPA-5 did not extract any protein from *R. capsulatus* membranes. MPA-2 extracted a moderate amount of intact LHI–RC superassembly from the native membrane (Supporting Information), but MPA-2 was substantially less effective at superassembly solubilization than TPA-2. Variation of alkyl-chain length among MPA-2 analogues showed that the 12-carbon length is optimal: analogues that contained 8- or 10-carbon segments extracted only very small amounts of LHI–RC superassembly, while analogues that contained 14- or 16-carbon segments extracted no protein at all (Supporting Information). Overall, the most effective monopod amphiphile we found, MPA-2, featured a branched hydrophilic group, which raises the possibility that branched hydrophilic groups will have general utility in the development of new detergents. The most important conclusion from the MPA studies, however, is that the most effective compound in this series is markedly inferior to glycotripod amphiphile, TPA-2, which suggests the existence of synergy between branching in the lipophilic and hydrophilic portions.

The presence of an aromatic ring in TPA-2 could be problematic for work with membrane proteins that do not absorb strongly in the visible or near-IR region. Therefore, we examined the saturated analogue TPA-2-S, which lacks a strong UV chromophore. Orange OT solubilization indicated a CMC of 1.8 mM. We were delighted to find that TPA-2-S is at least as effective as TPA-2, if not slightly superior, at extracting intact LHI–RC superassembly from *R. capsulatus* membranes (Supporting Information).

Comparison of TPA-2 and TPA-2-S to standard detergents that are used as tools for photosynthetic superassembly solubilization and stabilization revealed clear advantages for the glycotripod amphiphiles. In a separate effort, we have evaluated >120 conventional detergents with the *R. capsulatus* system.[12] Dodecylmaltoside (DDM) emerged as one of the most effective detergents, which is consistent with the widespread use of DDM for structural and functional studies of membrane proteins. DDM was comparable to TPA-2 and TPA-2-S in terms of LHI–RC superassembly extraction efficiency; however, a substantial distinction between conventional and glycotripod architectures became apparent when we examined the stability of the solubilized superassembly. Stability was monitored by following the 875/680 nm absorption ratio over a few weeks (absorption at 680 nm arises from oxidation of bacteriochlorophyll that has dissociated from LHI protein). As shown in Figure 2, LHI–RC superassembly solubilized with



TPA-2-S

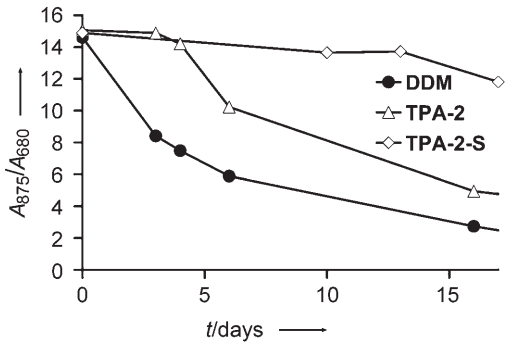


Figure 2. To monitor the ability of micelles of dodecylmaltoside (DDM), TPA-2, and TPA-2-S to stabilize membrane-protein complexes, spectra of the protein purified by each detergent were recorded as a function of time. The initial spectrum ($t = 0$) was acquired directly after elution of the protein from affinity chromatography. The integrity of the LHI–RC superassembly solubilized by these relatively mild detergents was monitored quantitatively from a scatter-corrected absorbance ratio (A_{875}/A_{680}). The ratio of a sample of completely folded and functional superassembly was >14.5, and this ratio declined dramatically as the multisubunit complex disassembled and denatured.

DDM began to degrade immediately when incubated at room temperature. In contrast, LHI–RC superassembly solubilized with TPA-2 remained stable for several days, but then degraded. TPA-2-S displayed the most promising behavior: even after two weeks, most of the LHI–RC superassembly was intact. Stability on this time scale is important for physical characterization and ultimately for crystallization or NMR spectroscopy-based structural studies.

The visible–near-IR absorbance data indicate that LHI–RC superassembly freshly solubilized with DDM, TPA-2, or TPA-2-S is intact; however, we used time-resolved absorbance measure-

ments as a further probe for native-like structure and function. Photon absorption by LHI initiates a series of excited state energy-transfer and electron-transfer reactions that culminate in the formation of a charge-separated state in the RC that is comprised of a porphyrin radical cation and a quinone radical anion at the B site in the RC complex ($P^+Q_B^-$). This RC state, $P^+Q_B^-$, in which the charges are separated by ~30 Å (nearly the width of a lipid bilayer), is easily identified by its characteristic recombination lifetime, which is determined by the rate of regeneration of the RC ground state, in the absence of exogenous donor and acceptor molecules. The lifetime for $P^+Q_B^-$ state decay is 2.1 sec for superassembly solubilized by either TPA-2 or DDM and 1.5 sec for superassembly solubilized by TPA-2-S (Supporting Information). These results indicate that native excited state energy- and electron-transfer pathways are intact in each case, with minor variations among amphiphiles.

We have demonstrated the importance of branching in the hydrophilic portion of tripod amphiphiles, and we have shown how this new feature complements branching in the lipophilic portion to generate optimal behavior toward a delicate protein superassembly from *R. capsulatus* membranes. The best new amphiphiles, TPA-2 and TPA-2-S, are clearly superior to conventional biochemical detergents with regard to long-term stability of solubilized LHI–RC superassembly. It is unlikely that any single amphiphile will be a "magic bullet" for membrane proteins; however, the ability to tune properties by modification of both lipophilic and hydrophilic portions, as illustrated here, suggests that glycotripod amphiphiles can be a productive source of tools for membrane-protein science. The branched carbohydrate units we have introduced could be generally useful for development of new biochemical detergents, since even among the conventional MPA series the best characteristics were displayed by a branched head-group (MPA-2). Overall, the TPA vs. MPA or DDM comparisons indicate that the new amphiphile design strategies we have introduced can produce useful alternatives to conventional detergents for membrane-protein manipulation.

Experimental Section

Details can be found in the Supporting Information.

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Keywords: amphiphiles · detergent design · membrane proteins · solubilization · stabilization

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