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Crystallographic Characterization of *N*-Oxide Tripod Amphiphiles

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Abstract: Tripod amphiphiles are designed to promote the solubilization and stabilization of intrinsic membrane proteins in aqueous solution; facilitation of crystallization is a long-range goal. Membrane proteins are subjects of extensive interest because of their critical biological roles, but proteins of this type can be difficult to study because of their low solubility in water. The nonionic detergents that are typically used to achieve solubility can have the unintended effect of causing protein denaturation. Tripod amphiphiles differ from conventional detergents in that the lipophilic segment contains a branchpoint, and previous work has shown that this unusual amphiphilic architecture can be advantageous relative to traditional detergent structures. Here, we report the crystal structures of several tripod amphiphiles that contain an *N*-oxide hydrophilic group. The data suggest that tripods can adapt themselves to a nonpolar surface by altering the hydrophobic appendage that projects toward that surface and their overall orientation relative to that surface. Although it is not possible to draw firm conclusions regarding amphiphile association in solution from crystallographic data, trends observed among the packing patterns reported here suggest design strategies to be implemented in future studies.

Introduction

Membrane proteins constitute a substantial fraction of the human proteome, and many members of this protein class are known to play crucial biological roles (e.g., receptors, ion channels).[1] Despite the prevalence and importance of membrane proteins, however, structural and functional analysis of these biomacromolecules is far less developed than for soluble proteins.[2] The traditional approach to protein characterization begins with purification from a biological source, followed by studies of solutions with controlled composition that contain the protein and as little else as possible. One common goal is to obtain high-resolution structural information, via NMR or via crystallization followed by X-ray diffraction; crystal growth usually requires homogeneous solutions of pure protein.[3] Extending the traditional approach to membrane proteins is challenging because these molecules have evolved to reside in a lipid bilayer, which is a highly asymmetric environment, rather than in aqueous solution.[4] Extraction of intrinsic proteins from biological membranes requires the use of small amphiphilic molecules, generally detergents, as do all subsequent manipulations of the protein sample.[5] Even the crystallization of these proteins is conducted in the presence of amphiphilic additives, which are usually incorporated into the resulting crystals.[6] The amphiphiles are intended to cover the large hydrophobic patches found on the surfaces of membrane proteins in their native states, surfaces that are normally embedded in the lipophilic core of the membrane.[7]

It can be quite demanding to identify an amphiphile that effectively solubilizes a membrane protein of interest in a native-like state, because the qualities that enable amphiphiles to cover exposed lipophilic side chains on the protein also enable the amphiphiles to interact favorably with lipophilic side chains that would be buried in the core of the folded protein. Thus, detergents often induce protein unfolding.[5a,b,8] Extensive empirical study has identified a few guidelines for selecting "mild" detergents; the most general guideline is avoidance of ionic detergents (e.g., SDS, CTAB). Membrane protein researchers generally favor detergents with uncharged polar headgroups,[5d,9] popular examples of which include octyl glucoside (OG),[5a] dodecyl maltoside (DDM),[10] lauryldimethylamine oxide

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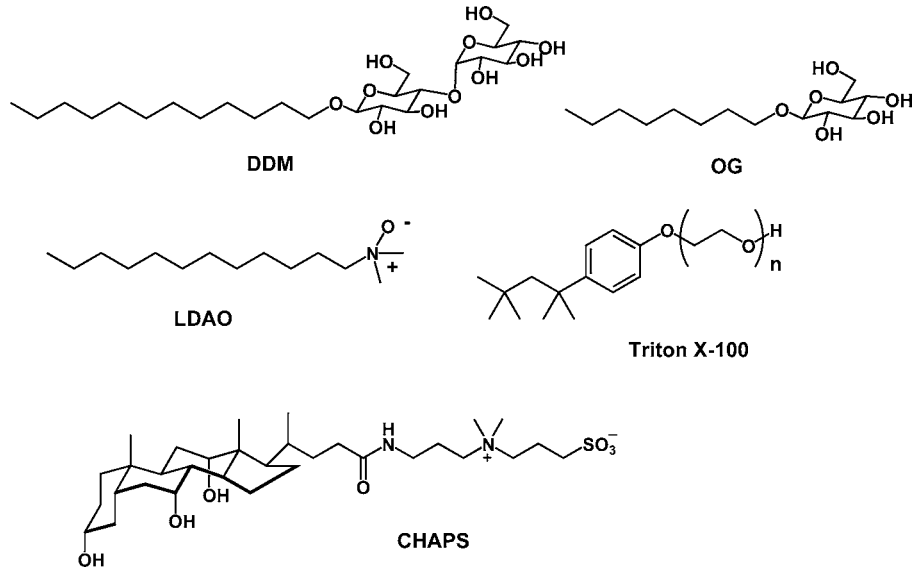

Figure 1. Chemical structures of widely used detergents for membrane protein studies.

(LDAO),[11] Triton X-100,[12] and CHAPS[13] (Figure 1). A detergent that is effective for extraction of an intrinsic protein from its membrane frequently turns out not to be ideal for subsequent characterization; in these cases, a detergent exchange procedure is employed after the initial isolation.[14] Thus, for example, Triton X-100 is often effective for disruption of a biological membrane and concomitant "capture" of embedded proteins in a native-like state, but this detergent has generally not been amenable to crystallization.[15] Therefore, a sample solubilized with Triton X-100 can have the detergent component exchanged for more crystallization-prone examples such as DDM, OG, or LDAO, which may not have been capable of extracting the protein from its native membrane.[16]

Most biochemical detergents share a simple architecture: a polar "headgroup" is placed at one end of a long alkyl "tail" (as illustrated by DDM, OG, and LDAO). A few common examples, such as Triton X-100 and CHAPS, deviate from this structural pattern, and the widespread use of these atypical detergents suggests that it might be fruitful to explore a broader range of variations in amphiphilic architecture. To date, only a few efforts of this type have been reported.[17] Such research efforts can be difficult to pursue because they require both expertise in the synthesis and characterization of organic molecules and expertise in the biochemistry of membrane proteins, skill sets that generally do not overlap.

Our interest in the design of new amphiphiles for solubilization and ultimately crystallization of membrane proteins has focused on a simple strategy for modulating conformational mobility.[18] The conventional detergents most commonly employed in successful membrane protein crystallization efforts, DDM, OG, and LDAO, all contain linear alkyl groups, which are highly flexible.[15] Because membrane protein crystallization remains notoriously difficult, even when these detergents are used, we wondered whether the inherent flexibility of linear alkyl groups works against formation of a crystalline lattice.[18] The rarity of membrane protein crystals grown in the presence of CHAPS or other rigid amphiphiles suggests that some degree of flexibility may be important in the amphiphile structure. Therefore, we have designed, synthesized, and evaluated new amphiphiles that contain at least one branchpoint.[18] This feature, a carbon atom bearing three or four non-hydrogen bonding partners, limits conformational mobility about nearby single

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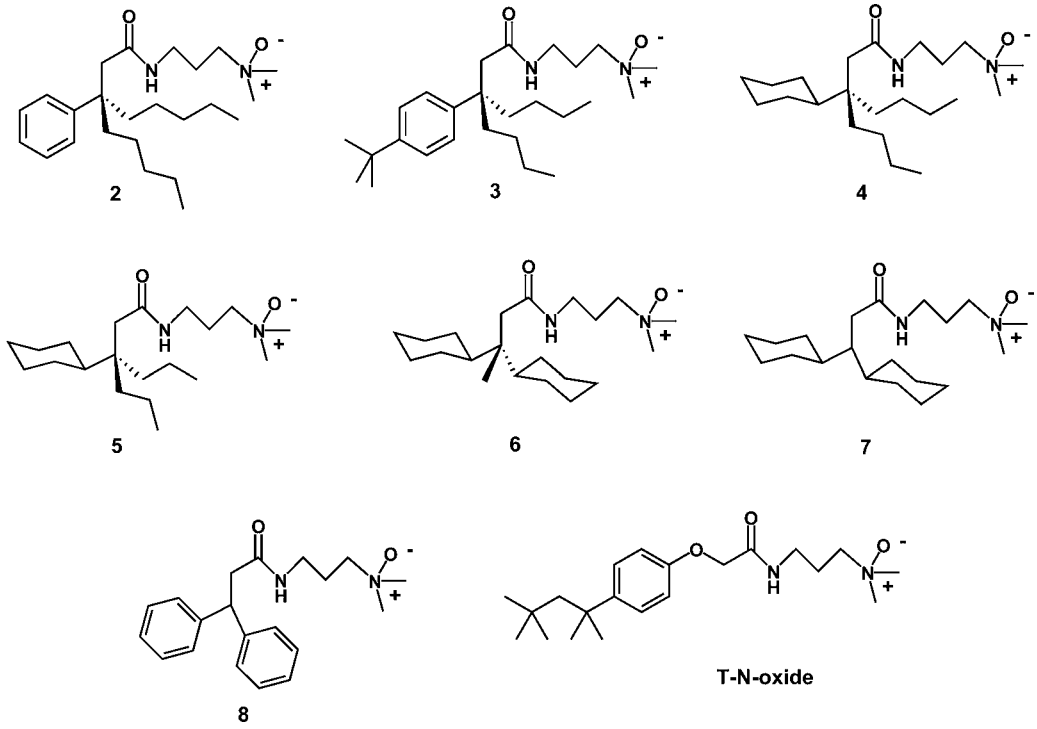
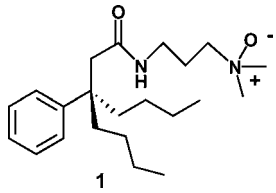


Figure 2. Chemical structures of amphiphiles.

bonds[19] but does not achieve the level of rigidity induced by a multicyclic skeleton such as that found in CHAPS. Our approach is illustrated by structure **1**, which contains a quaternary carbon. Because the quaternary carbon bears three hydrophobic appendages, along with a polar appendage, we refer to molecules of this type as "tripod amphiphiles". This design is modular; the size and conformational features of each hydrophobic appendage and of the hydrophilic appendage can be individually varied to tune amphiphile behavior. Tripod amphiphiles can be viewed as species that combine features of both conventional, highly flexible detergents and "facial amphiphiles", as identified by Kahne et al.[20]



We have previously shown that **1** can extract bacteriorhodopsin (bR) from the "purple membrane" of *Halobacterium salinarum*, and rhodopsin (Rho) from membrane preparations derived from bovine retinas.[18a,b] The solubilized states of both proteins are stable for several weeks.[18b] Both bR and a potassium channel from *Streptomyces lividans* have been crystallized from solutions in which the protein is solubilized by **1**, although structures were not determined.[18a,21] The behavior of **1** toward bR and Rho was shown to be superior to that of a related conventional detergent (LDAO) and to that of a handful of other tripod *N*-oxides. Tripod amphiphile **1** is now commercially available under the name "Tripao".

It would be valuable to have molecular-level understanding regarding the manner in which detergents or other amphiphiles, such as **1**, associate with one another in solution and with lipophilic surfaces of membrane proteins. Unfortunately, this type of information is very difficult to acquire, given the evanescent nature of micellar structures.[22] Here, we report crystal structures for **1**, crystallized both from aqueous solution and from organic solvents, and crystal structures of several other tripod amphiphiles (**2**−**6**) as well as related dipod amphiphiles (**7**,**8**) and an *N*-oxide that bears the hydrophobic segment of Triton X-100 (which we designate "T-*N*-oxide") (Figure 2). Collectively, these structures offer insight on the ways in which hydrophobic tripods prefer to pack against one another and a basis for comparing tripod self-association with self-association of more traditional hydrophobic moieties found in conventional detergents. These structural data, necessarily acquired in the highly ordered environment of a crystal, may not be directly relevant to the loose associations that occur in aqueous solution; nevertheless, trends in the packing data are suggestive with regard to the ways in which tripod amphiphiles might interact

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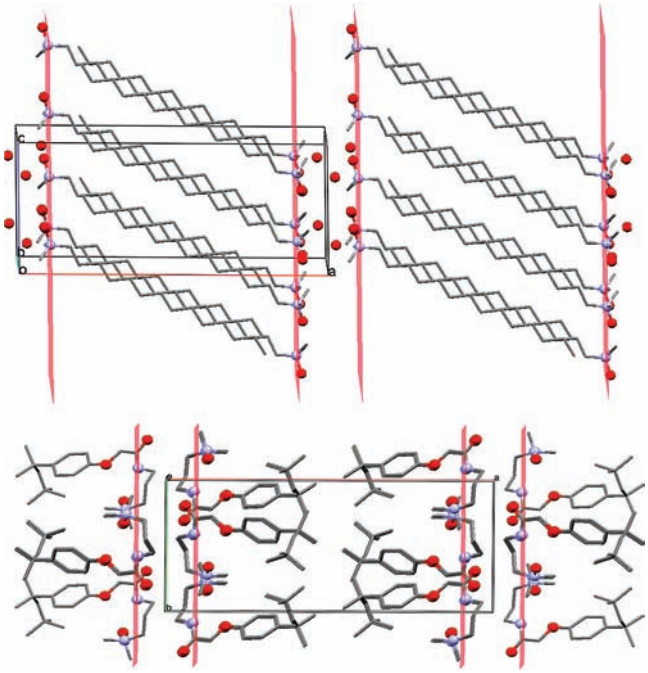

Figure 3. Packing patterns of TDAO (top; CSD code WURTIM) and T-*N*-oxide (bottom) in the crystalline state. The parallelepipeds represent the unit cells. The red lines represent the borders between polar and nonpolar layers.

with hydrophobic surfaces in aqueous solution and may therefore provide a useful basis for future designs.

Results and Discussion

The crystal structure of a conventional detergent bearing the *N*-oxide headgroup, tetradecylamine oxide (TDAO), is available in the Cambridge Structure Database[23] (CSD code WURTIM) and provides a useful point of reference for consideration of the tripod amphiphile structures reported here. TDAO is a slightly larger homologue of LDAO. The molecular packing in this crystal (Figure 3, upper part) shows alternating layers of polar moieties (the *N*-oxide groups) and nonpolar moieties (the alkyl groups, which adopt a fully extended conformation). This spatial arrangement maximizes polar−polar and nonpolar−nonpolar contacts and minimizes polar−nonpolar contacts. The polar−nonpolar layers arise because the molecules themselves are arranged in sheets in which all molecules are parallel to one another, and the molecules in adjacent sheets have antiparallel orientation relative to one another. The nonpolar layer is formed by interleaved alkyl groups from two layers of TDAO molecules. The nonpolar layer thickness can be defined as the distance from a nitrogen in one molecular sheet to the nearest nitrogen in the other sheet; this distance is 18.1 Å in the crystal of TDAO.

Figure 3 (lower part) shows the packing pattern in crystals of T-*N*-oxide grown from CH_2Cl_2/Et_2O. The pattern of alternating polar and nonpolar layers is analogous to that observed for TDAO. The polar group of T-*N*-oxide is identical to the polar group featured in all of the tripod amphiphiles discussed below: the *N*-oxide nitrogen is connected via a trimethylene unit to the nitrogen of a secondary amide group. Within the polar layers of crystalline T-*N*-oxide, each *N*-oxide oxygen forms an intramolecular H-bond to the amide NH. Each nonpolar layer in this crystal is formed by the tail-to-tail packing of two sheets

Table 1. Thickness of the Nonpolar and Polar Layers in the Crystal Structures of Amphiphiles

compound	nonpolar layer (Å)[a]	polar layer (Å)[a]	solvent[b]
TDAO·$2H_2O$	18.12[c]	4.66[c]	water
1·$2.5H_2O$	12.13	11.70	water
1·$0.5H_2O$	9.25	5.94	DCM/Et_2O
2	10.38	1.32	DCM/Et_2O
3	13.53	3.39	DCM/Et_2O
4	12.15	2.82	DCM/Et_2O
5	10.20	1.15	DCM/Et_2O
6·$3H_2O$	10.00	12.75	DCM/Et_2O
7	12.65	3.86	DCM/Et_2O
8	9.59	3.94	DCM/Et_2O
T-*N*-oxide	17.7	3.96	DCM/Et_2O

[a] Determined with carbonyl carbon atom, except for TDAO. [b] Solvent system used for crystal growth. [c] Determined with the nitrogen atom.

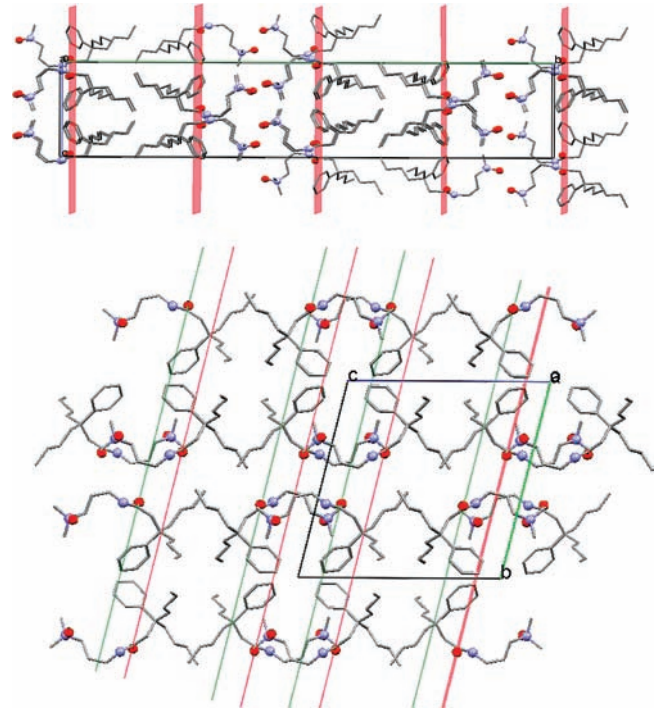

Figure 4. The lattices observed for tripod amphiphile **1** crystallized from water (top) or from organic solvents (bottom). The parallelepipeds represent the unit cells. In the upper image, the red lines represent the borders between polar and nonpolar layers. In the lower image, alternating red and green lines are used to represent the borders between polar and nonpolar layers, because in this structure there are two independent molecules of **1** in the lattice. Layer thicknesses given in Table 1 are based on separations between red lines, or separations between green lines.

of T-*N*-oxide molecules. Defining a nonpolar layer thickness requires one to identify a point of reference within the polar portion of the amphiphile. For TDAO, the nitrogen atom was an obvious point of reference, but for T-*N*-oxide (and the amphiphiles discussed below), the choice is less clear. We use the carbonyl carbon atom as the reference point for determining nonpolar layer thicknesses for T-*N*-oxide and amphiphiles **1**−**8** (Table 1). The nonpolar layer thickness in crystalline T-*N*-oxide is 17.7 Å, which is quite similar to that found for TDAO.

The tripod and dipod amphiphile crystal structures (Figures 4−6) display packing patterns that feature alternating polar and nonpolar layers, as seen for detergents TDAO and T-*N*-oxide (Figure 3). The nonpolar layers in the tripod and dipod amphiphile crystals are formed by two sheets of molecules that approach one another in tail-to-tail fashion. Within the tripod or dipod segments, that is, the segments up to the carbonyl

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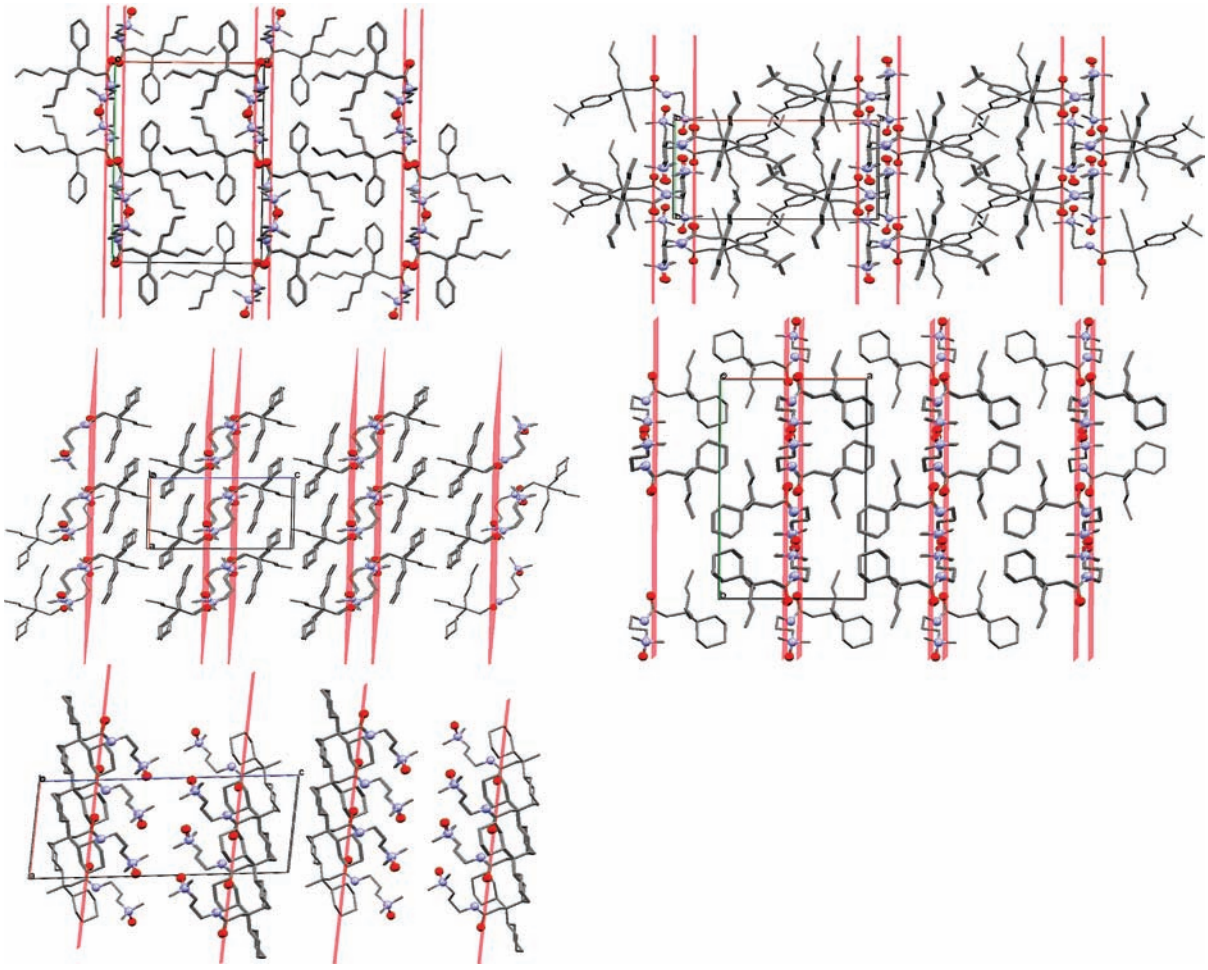

Figure 5. Crystal structures of tripod amphiphiles **2** (top left), **3** (top right), **4** (middle left), **5** (middle right), and **6** (bottom left). The parallelepipeds represent the unit cells. The red lines represent the borders between polar and nonpolar layers.

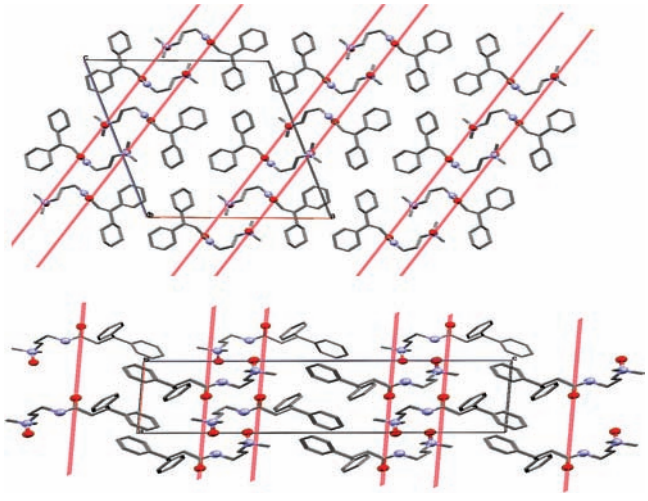

Figure 6. Crystal structures of dipod amphiphiles **7** (top) and **8** (bottom). The parallelepipeds represent the unit cells. The red lines represent the borders between polar and nonpolar layers.

carbon, the conformations observed appear to be those expected to have the lowest conformational energy. All CH_2-CH_2 bonds that can adopt an anti torsion angle do so, and the cyclohexyl units in **4**−**7** display chair conformations with substituents in equatorial positions (Figures 5 and 6).

The polar segments common to **1**−**8** and T-*N*-oxide, $C(=O)CH_2CH_2CH_2N(\rightarrow O)(CH_3)_2$, display considerable conformational variation. Presumably this variation arises at least in part from the drive to satisfy H-bonding potential. In a few cases, potential H-bonding groups include water molecules. One of the crystalline forms of **1** was grown from aqueous solution and contains 2.5 water molecules per amphiphile molecule. All other tripod crystals were grown from CH_2Cl_2/Et_2O, and most do not contain any water molecules; however, the crystal of **1** grown from CH_2Cl_2/Et_2O contains 0.5 water molecule per amphiphile, and the crystal of **6** contains 3 water molecules per amphiphile. In many cases, the trimethylene unit within the polar segment contains a gauche torsion angle. Several different H-bonding patterns are seen, including intramolecular N−O--H−N (as in T-*N*-oxide), and intermolecular N−O--H−N, H_2O--H−N, and C=O--H−N. The variations in the polar group conformation and polar group arrangement motivated us to use the carbonyl carbon rather than the *N*-oxide nitrogen as the point of reference for determining nonpolar layer thicknesses in these crystals (Table 1).

The packing of tripod segments within the nonpolar layers in the crystals of tripod amphiphiles **1**−**6** appears to display a common trend (Figures 4 and 5). In each case, two of the three nonpolar groups attached to the quaternary carbon lie approximately parallel to the plane of the layer. These two groups fill the space between neighbors within each sheet of molecules. The third nonpolar group projects into the nonpolar layer, toward

the other sheet of molecules that participates in this layer. The orientation of this "projecting" nonpolar group relative to the plane of the nonpolar layer varies from structure to structure. In **2** and **4**, the projecting nonpolar group (butyl in both cases) is approximately perpendicular to the plane of the nonpolar layer; this orientation is particularly noticeable for **2** (Figure 5). In most other cases, the projecting nonpolar group has an oblique orientation relative to the plane of the nonpolar layer. This projecting group is butyl for both crystal forms of **1**, *p*-*t*-butylphenyl for **3**, cyclohexyl for **5**, and methyl for **6**.

In contrast to the seven structures for tripod amphiphiles, we have only two for dipod amphiphiles (**7** and **8**; Figure 6). The packing trend noted above seems to hold for **8** in that one phenyl group lies approximately parallel to the plane of the nonpolar layer, while the other phenyl group projects obliquely into the nonpolar layer. In contrast, for **7** both of the cyclohexyl rings seem to project obliquely, but at a shallow angle, into the nonpolar layer.

It is impossible to draw firm conclusions regarding the ways in which tripod amphiphiles self-associate in aqueous solution based on the packing patterns observed in the crystals of **1**−**6**, or regarding the ways that these amphiphiles might arrange themselves around nonpolar surfaces displayed by a membrane protein; nevertheless, we believe that the trends observed in these crystal structures offer a basis for speculation on these important issues. The seven tripod amphiphile packing patterns display a range of nonpolar larger thicknesses, from 9.3 to 13.5 Å. These nonpolar layers are consistently thinner than those formed by the detergent with a conventional amphiphilic architecture, TDAO, or T-*N*-oxide. Consideration of the seven packing patterns collectively suggests that the tripod architecture offers flexibility in the way that a collection of these molecules might adapt themselves to a nonpolar surface. The data suggest that it might be possible to optimize such interactions by changing the identities of the nonpolar units that are directed toward neighboring amphiphiles and toward the surface and/or by changing the "tilt" of individual tripod units. The tilt of a tripod can be defined on the basis of the plane that contains the carbon atoms from the three nonpolar units that are directly bonded to the quaternary carbon, and the mean plane of the nonpolar surface with which the tripod amphiphile interacts; the tilt is the angle between these two planes. The ways in which a tripod amphiphile layer accommodates itself to the hydrophobic surface displayed by another amphiphile layer in these crystals may bear some resemblance to the ways in which clusters of tripod amphiphiles in solution accommodate themselves to hydrophobic surfaces displayed by membrane proteins.

Comparison of the two forms of **1** seems to offer a hint of the way in which adjustments proposed in the previous paragraph might be realized. The form crystallized from water has a nonpolar layer thickness of 12.1 Å, while the crystal from organic solvents has a 9.3 Å nonpolar layer thickness. This range of nonpolar thicknesses spans almost the entire range observed among crystalline **1**−**6** (only **3**, with a 13.5 Å nonpolar layer thickness, lies outside this range). The packing pattern of **1** as crystallized from organic solvents is more complex than the other packing patterns presented here, because there are two independent molecules of **1** in this crystal. This complexity makes it more challenging to define the border between polar and nonpolar layers in this form of **1** relative to the other crystals. The lattice view in the lower part of Figure 4 suggests that there is an alternative way to define the layer thicknesses in this crystal, relative to the method used to generate the values reported in Table 1. Thus, a nonpolar layer in this crystal of **1** could be defined by the leftmost red line and the second green line from the left, and the corresponding polar layer could be defined by the second green line from the left and the third red line from the left. In this case, the nonpolar layer thickness is 6.96 Å and the polar layer thickness is 8.23 Å; this nonpolar layer thickness would be by far the smallest among the structures considered. The origin of the difference between the packing of **1** in the two crystal forms is impossible to identify by inspection, but perhaps one important factor is the angle at which butyl groups project into the nonpolar layers. Overall, the differences between the two packing patterns displayed by **1** suggest that variations in relative positioning of neighboring tripod amphiphiles allow these molecules to accommodate themselves in diverse ways to nonpolar surfaces.

Table 2. bR Solubilization Efficiency of the Amphiphiles **1**, **5**, **7**, and T-*N*-Oxide

	1	**5**	**7**	T-*N*-oxide
CMC (mM)	4.4	4.5	4.9	1.1
concentration (mM)	8.8	9	9.8	8.9
concentration (%)	0.32	0.31	0.33	0.33
solubilization yield (%)	87	91	70	50

As a complement to the crystallographic results presented above, we examined the new amphiphiles for the ability to extract bR from its native membrane environment. Many of the new amphiphiles (**2**, **3**, **4**, and **6**) were not sufficiently soluble in aqueous buffer to support extraction studies; amphiphile **8** proved to be too soluble for this purpose (CMC = 78 mM). We used **1** as a positive control, because this tripod has previously been shown to extract bR efficiently.[18a,b] Results are summarized in Table 2. Critical micelle concentration (CMC) values were determined for **5**, **7**, and T-*N*-oxide via fluorescent dye solubilization, as previously described.[24] Solubilization of bR by **1**, **5**, or **7** was evaluated at an amphiphile concentration of 2 × CMC; because the CMC values are quite similar for these three compounds, the amphiphile concentrations in the extraction solutions were comparable, in terms of molarity or weight %. T-*N*-oxide was examined at an absolute concentration similar to that of the other compounds, even though this detergent has a somewhat lower CMC than does **1**, **5**, or **7**.

The data in Table 2 show that tripod amphiphile **5** is comparable to **1** in its ability to extract intact bR from the native membrane. Dipod amphiphile **7** is less effective, and detergent T-*N*-oxide displays a further decrease in efficacy. Previous work has shown that the conventional detergent LDAO causes bR denaturation upon attempted solubilization.[18a]

Conclusions

We have presented a set of crystal structures that show how tripod amphiphiles assemble into a regular lattice. Trends observed among these packing patterns are suggestive with regard to the way such amphiphiles may associate with exposed hydrophobic surfaces of membrane proteins. In particular, the data raise the possibility that tripod amphiphiles can accommodate themselves to a nonpolar surface by altering their overall tilt relative to that surface, and by varying which of the nonpolar appendages is oriented toward the surface. These observations help to identify new design strategies. For example, the ability of tripod amphiphiles to solubilize membrane proteins in their native states may be optimized when the three hydrophobic

(24) Chattopadhyay, A.; London, E. *Anal. Biochem.* **1984**, *139*, 408–412.

appendages differ from one another, which could enhance the ability of a tripod layer to adapt to local topological variations in the protein surface. Examples explored to date have had at least two identical hydrophobic appendages, and it will be interesting to evaluate new tripod amphiphiles with more diverse sets of hydrophobic groups.

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Supporting Information Available: Synthetic protocols and characterization data for new compounds. This material is available free of charge via the Internet at http://pubs.acs.org.

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